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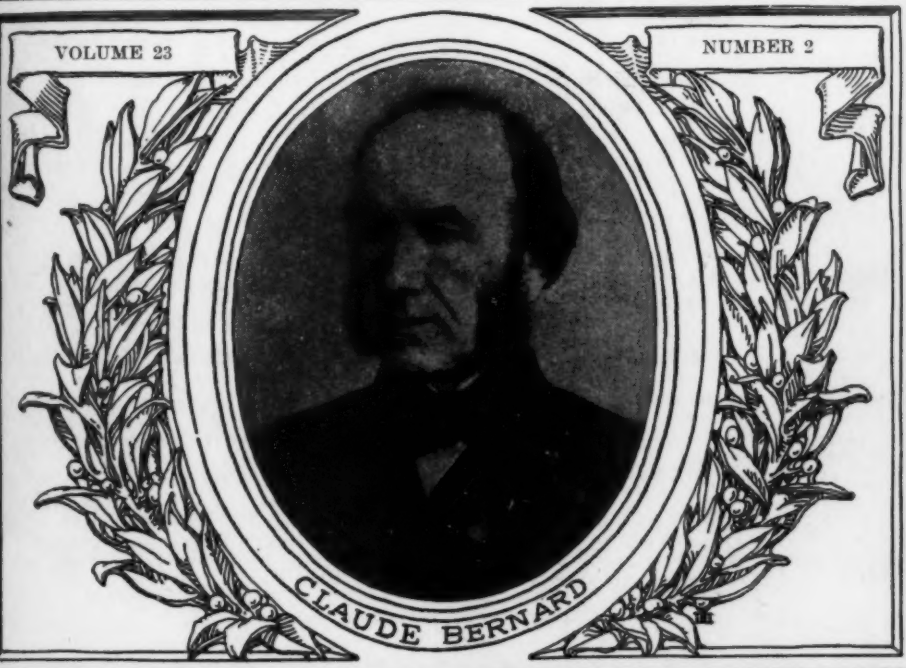


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# THE EFFECT OF ADDED GLUCOSE UPON THE DIGESTIBILITY OF PROTEIN AND OF FIBER IN RATIONS FOR SHEEP [1]

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(Received for publication July 18, 1941)

During a recent investigation in this laboratory (Mitchell and Hamilton, '40) concerned with the utilization of the energy of rations containing varying percentages of protein and of glucose when fed to beef calves, it was found that increasing the gross energy of rations by about 30% by the addition of glucose failed to increase their metabolizable energy contents (expressed in calories per kilogram of dry matter consumed). When the metabolizable and net energy values of glucose were calculated by the usual indirect procedure, these values were found to be considerably lower than might be expected. The average of twelve estimates indicated that glucose [2], with a gross energy value of 3680 calories per kilogram of dry matter, possessed on the same basis 2401 calories of metabolizable and 1175 calories of net energy when fed to beef calves.

Similar low metabolizable and net energy values for starch, when fed to steers, have been obtained by Armsby and Fries ('18) and by Kellner and Köhler ('00).

The failure to increase the metabolizable energy value of a ration, when the proportion of the so-called available carbohydrate fraction is increased over the so-called non-available carbohydrate fraction, at first may seem paradoxical. How-

[1] Aided by the Works Progress Administration, Project No. 80057.

[2] Cerelose. Kindly supplied by The Corn Products Refining Company.



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ever, it is a well-known fact that the addition of starch or molasses to a ration for ruminants usually decreases the digestibility of the nutrients in the basal ration, this being particularly noticeable in case of protein and of crude fiber (cf. Kellner, '09; Lindsey and Smith, '10; Ewing and Wells, '15; Armsby and Fries, '18; Briggs, '37; and Scharrer and Nebelsiek, '38). However, some data have been interpreted to indicate a more favorable effect of soluble sugars added to rations for ruminants (Patterson and Outwater, '07, and Williams, '25).

In four trials with beef calves, Mitchell and Hamilton ('40) found that corn sugar, added to rations containing varying percentages of protein, decreased the digestibility of the crude fiber by about 25%. In order to test more completely the effects of adding corn sugar to rations when fed to ruminants, the experiments described below were carried out.

## PLAN OF EXPERIMENT

The plan of the experiment was to determine the digestibility of the nutrients and the metabolizable energy in rations with and without added glucose. Six cross-bred ewe lambs, weighing between 60 and 100 pounds, were used as experimental animals. The rations used were of the same composition as those fed to steers in a previous experiment (Mitchell and Hamilton, '40). The basal ration consisted of cut timothy hay, ground yellow corn, and cottonseed meal in the approximate ratios of 2:2:1. The ration containing the glucose, hereafter referred to as the sugar ration, was the same as the basal except that glucose was added to the extent of 20 to 30% — the exact amount of sugar added depended upon the avidity with which the individual sheep ate the ration with the added sugar. The plan of feeding involved a double reversal procedure. The basal ration was fed at a level calculated to just maintain live weight. The food maintenance requirement for these sheep was assumed to be adequately covered by 1.64 pounds of this particular ration per 100 pounds live weight. When receiving the sugar rations, each sheep

received exactly the same amount of the basal ration as she did in the no-sugar period plus 150 to 200 gm. of the corn sugar daily. In addition, in each period each sheep received daily 6 gm. bone meal, 3 gm. yeast, 3 gm. salt, and 0.5 cc. cod liver oil. While six sheep were used and the plan was to carry out a double reversal procedure on each, it was possible to obtain such data on but four of the animals. In one case data were obtained on the basal ration in one period and on the sugar ration in one period. In another case the sheep would not eat the basal ration so data were obtained on the sugar ration for one period only.

The apparatus consisted of six digestion crates which have been used in this laboratory for a number of years (cf. Keilholz, '26). Daily collections of urine and feces were made for 10 days and each collection period was preceded by a 7-day preliminary feeding period. Feces samples were preserved by drying at 50 to 60° C. and urine samples were preserved with toluene. Methods of analysis were those of the A. O. A. C. ('40). Gross energy of feeds, feces, and urines was determined by a Parr Oxygen bomb calorimeter. The urine samples were evaporated on cellulose blocks before burning. The metabolizable energy of the rations was calculated by subtracting, from the gross energy consumed, the sum of the gross energy of the feces, urine, and methane.[3] The energy in the urine was corrected for nitrogen balance according to Armsby's ('17, p. 641) procedure, using Rubner's (1885) factor of 7.45 Calories for each gram of nitrogen balance.

## RESULTS

The essential collection data are tabulated in table 1. The average composition of the basal and of the sugar rations are, respectively, 91.6 and 91.7% dry matter, 14.6 and 13.1% crude protein, 15.5 and 11.8% crude fiber, 53.0 and 62.1% nitrogen-

[3] The methane was calculated by the use of Armsby's factor of 4.5 gm. of methane per 100 gm. of digestible carbohydrates. This factor may not be strictly applicable to sheep, but, since the rations were similar in most respects, the errors probably are not serious.

TABLE 1

*Collection data.*

| SHEEP | BREED | WEIGHT | | DAILY FEED [1] | | | | AVERAGE DAILY ORTS | EXCRETED DAILY | | PERIOD |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Start | End | Timothy hay | Corn | Cotton-seed meal | Sugar | | Feces (dry) | Urine and washings | |
| | | *lb.* | *lb.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | |
| 554 | Hampshire Cross | 57.0 | 58.5 | 182 | 182 | 102 | .... | .... | 149 | 4009 | I [3] |
| 537 | " " | 73.0 | 74.5 | 224 | 224 | 126 | .... | .... | 181 | 2357 | " |
| 822 | Southdown Cross | 91.5 | 94.5 | 264 | 264 | 148 | .... | .... | 216 | 2647 | " |
| 882 | " " | 75.0 | 76.5 | 224 | 224 | 126 | .... | .... | 181 | 2466 | " |
| 805 [2] | " " | 79.5 | 78.0 | 238 | 238 | 134 | .... | 161 | 165 | 2395 | " |
| 554 | Hampshire Cross | 62.5 | 63.0 | 182 | 182 | 102 | 150 | .... | 191 | 4027 | II [4] |
| 537 | " " | 80.5 | 81.5 | 224 | 224 | 126 | 200 | .... | 237 | 2621 | " |
| 540 [2] | " " | 80.0 | 80.5 | 184 | 184 | 104 | 200 | 33 | 174 | 2690 | " |
| 822 | Southdown Cross | 98.0 | 100.5 | 264 | 264 | 148 | 200 | .... | 256 | 2880 | " |
| 882 | " " | 80.0 | 83.0 | 224 | 224 | 126 | 200 | .... | 211 | 2627 | " |
| 805 [2] | " " | 81.0 | 82.5 | 238 | 238 | 134 | 150 | 94 | 246 | 2584 | " |
| 554 | Hampshire Cross | 61.5 | 62.0 | 182 | 182 | 102 | .... | .... | 150 | 4616 | III [5] |
| 537 | " " | 80.5 | 81.0 | 224 | 224 | 126 | .... | .... | 182 | 3024 | " |
| 822 | Southdown Cross | 99.5 | 99.5 | 264 | 264 | 148 | .... | .... | 209 | 3183 | " |
| 882 | " " | 81.5 | 82.0 | 224 | 224 | 126 | .... | .... | 178 | 2652 | " |

[1] Each sheep was fed at the daily rate of 1.64 pounds per 100 pounds live weight. In addition to the experimental ration each sheep received daily 6 gm. bone meal, 3 gm. yeast, 3 gm. salt, and 1 cc. cod liver oil.

[2] Orts in this case. Nutrients in orts accounted for.

[3] Period I — January 30 to February 8.

[4] Period II — February 20 to March 1.

[5] Period III — March 8 to March 17.

free extract, 4.2 and 3.4% ether extract, 4.3 and 3.3% ash, and 4.13 and 3.94 cals. of gross energy per gram. The apparent digestion coefficients, the total digestible nutrients, and the metabolizable energy contents of the two rations are tabulated in table 2. According to these data, the addition of sugar increased the apparent digestibility of dry substance, N-free extract, and total carbohydrates, but decreased the apparent digestibility of total nitrogen and crude fiber as well as the metabolizable energy content.

Perhaps a better way of assessing the significance of these differences is to compare the values obtained on each sheep and with the animals consuming the two different rations. This is possible in five of the six sheep used. In four cases the double reversal procedure was used and in these cases the two basal periods were averaged and this average compared with the sugar period. In one case there are data on one basal and one sugar period only. These comparisons are susceptible to statistical analysis of paired data according to Student's ('08) method. In table 3 these paired comparisons are summarized and the results of the statistical treatment are given. The P values or the odds given indicate the probability that chance alone caused differences as great as those obtained. Assuming that P values of 0.03 or less, or odds of 1:33 or more, are significant, then the following conclusions may be made with reference to the effect of the addition of corn sugar to the basal ration: (1) It caused an increase in the apparent digestibility of the dry matter, N-free extract, and total carbohydrates. (2) It caused a decrease in the apparent digestibility of total nitrogen and crude fiber, and a decrease in the metabolizable energy concentration. (3) The sugar ration contained an average of 1.7 pounds of T. D. N. more per 100 pounds than did the basal ration. The probability that this difference was fortuitous was 0.0307, a value that most would consider significant. (4) It caused no difference in the apparent digestibility of either ether extract or gross energy.

TABLE 2

*Coefficients of apparent digestibility by individual sheep.*

| SHEEP | RATION | PERIOD | DRY SUBSTANCE | TOTAL NITROGEN | CRUDE FIBER | ETHER EXTRACT | N-FREE EXTRACT | TOTAL CARBO-HYDRATES | GROSS ENERGY | TOTAL DIGESTIBLE NUTRIENTS | METABOLIZABLE ENERGY |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | % | % | % | % | % | % | % | % | Cal. per kg. Dry matter |
| 554 H | Basal | I | 66.2 | 62.4 | 48.2 | 69.2 | 77.0 | 70.4 | 65.7 | 63.7 | 2417 |
| | Basal + sugar | II | 67.7 | 56.9 | 28.7 | 73.5 | 79.0 | 70.9 | 65.6 | 64.5 | 2311 |
| | Basal | III | 66.4 | 61.2 | 45.8 | 72.1 | 76.8 | 69.9 | 65.9 | 63.8 | 2464 |
| 805 S | Basal | I | 59.9 | 53.8 | 36.5 | 73.8 | 73.7 | 65.1 | 62.8 | 58.5 | 2363 |
| | Basal + sugar | II | 61.1 | 41.0 | 29.9 | 68.8 | 75.2 | 67.2 | 60.0 | 59.0 | 2136 |
| 882 S | Basal | I | 66.6 | 67.0 | 44.1 | 75.3 | 76.7 | 69.2 | 66.9 | 64.1 | 2547 |
| | Basal + sugar | II | 71.9 | 58.5 | 37.7 | 79.2 | 83.1 | 76.0 | 70.2 | 68.9 | 2487 |
| | Basal | III | 67.7 | 63.6 | 49.1 | 76.7 | 78.2 | 71.7 | 68.3 | 65.7 | 2568 |
| 537 H | Basal | I | 66.6 | 66.1 | 40.6 | 80.5 | 76.3 | 68.0 | 66.5 | 63.7 | 2476 |
| | Basal + sugar | II | 68.3 | 56.6 | 26.0 | 69.0 | 80.1 | 71.6 | 65.0 | 64.7 | 2262 |
| | Basal | III | 67.0 | 63.6 | 42.9 | 74.1 | 76.6 | 69.1 | 66.5 | 63.7 | 2579 |
| 822 S | Basal | I | 66.1 | 65.9 | 44.3 | 74.9 | 76.7 | 69.2 | 66.7 | 63.9 | 2472 |
| | Basal + sugar | II | 69.7 | 57.4 | 37.4 | 76.0 | 81.1 | 73.9 | 67.9 | 67.0 | 2456 |
| | Basal | III | 67.6 | 61.4 | 50.2 | 72.8 | 78.1 | 71.9 | 67.9 | 65.2 | 2557 |
| 540 H | Basal + sugar | II | 71.9 | 53.1 | 36.5 | 74.8 | 82.6 | 76.1 | 69.0 | 67.8 | 2381 |
| | Average–Basal | (9) | 66.0 | 62.8 | 44.6 | 74.4 | 76.7 | 69.4 | 66.4 | 63.6 | 2494 |
| | Average–Basal + sugar | (6) | 68.4 | 53.9 | 32.7 | 73.6 | 80.2 | 72.6 | 66.3 | 65.3 | 2339 |

Since each sheep, when on the sugar ration, consumed the same, or very nearly the same, amount of the basal ration as she did when on the basal ration alone, any change in the digestibility of the nutrients in the sugar ration may be attributed directly to the added sugar. Thus by assuming that the added sugar was completely digested and that the digestibility of the nutrients in the basal ration remained unchanged by this addition, the total nutrients that should have been digested from the sugar ration may be calculated. These cal-

TABLE 3

*Summary and statistical analysis of coefficients when each sheep serves as her own control.*

| | AVERAGES FOR BASAL RATION | AVERAGES FOR BASAL + SUGAR RATION | DIFFERENCE IN FAVOR OF BASAL + SUGAR RATION | STANDARD DEVIATION | P VALUE [1] | ODDS |
|---|---|---|---|---|---|---|
| Dry matter, pct. | 65.4 | 67.7 | +2.36 | 1.36 | 0.0123 | 1: 79 |
| Total nitrogen, pct. | 61.9 | 54.1 | —7.78 | 2.69 | 0.0023 | 1: 435 |
| Crude fiber, pct. | 43.8 | 31.9 | —11.86 | 1.25 | 0.0001 | 1: 10,000 |
| Ether extract, pct. | 74.3 | 73.3 | —1.00 | 1.49 | 0.127 | 1: 8 |
| N-free extract, pct. | 76.4 | 79.7 | +3.34 | 1.46 | 0.005 | 1: 200 |
| Total carbohydrates, pct. | 68.9 | 71.9 | +3.00 | 1.59 | 0.0022 | 1: 454 |
| Gross energy, pct. | 66.0 | 65.7 | —0.26 | 0.23 | 0.335 | 1: 3 |
| Total digestible nutrients, pct. | 63.1 | 64.8 | +1.70 | 1.34 | 0.0307 | 1: 33 |
| Metabolizable energy, Cals. per kg. dry matter | 2480 | 2330 | —1.50 | 82.5 | 0.010 | 1: 100 |

[1] Probability or odds that the differences were fortuitous.

culations show that, on the assumption that the sugar had been completely digested, there was a decrease in the digestibility of all nutrients in the basal ration. These decreases amounted to 40.3 gm. of dry matter, 7.5 gm. crude protein, 10.5 gm. crude fiber, 0.2 gm. ether extract, 22.7 gm. of nitrogen-free extract, 33.4 gm. total carbohydrates, and 210 Calories of gross energy.

A decrease in digestibility of the energy and of the nitrogen-free extract could be accounted for by a decrease in the digestibility of these nutrients in either the basal part of the ration

or in the sugar or in both. However, the decrease in the digestibility of the nutrients not in the sugar, namely, protein, fiber and fat, can be accounted for only by a decrease in the digestibility of these nutrients in the basal part of the ration.

As Armsby ('17, p. 619) has suggested, a possible explanation for the decrease in the apparent digestibility of protein is the increase in metabolic nitrogen due to the added sugar. According to Harris and Mitchell ('41), sheep on a nearly nitrogen-free ration excrete 5.5 mg. of metabolic nitrogen in the feces per gram of dry food consumed. Using this factor, it may be calculated that the average true digestibility of the nitrogen of the basal ration was 81.8 and that of the basal plus sugar ration was 79.4. The difference of 2.4% was found to be not significant. Thus the significantly lower apparent digestibility of the nitrogen in sugar ration, as compared with that of the basal ration, is probably accounted for entirely by the sugar addition.

As to the marked decrease in the digestibility in the fiber in the basal ration when sugar is added, the most probable explanation seems to be that the microorganisms in the rumen, which, in the absence of a more soluble form of carbohydrates, attack the cellulose, prefer the sugar to the fiber. That this is apparently true is indicated from the following observations.

It may also be calculated that, if the sugar of the sugar ration had been completely absorbed and if the nutrients in the basal fraction of the sugar ration had been digested to the same extent as they were in the absence of sugar, 39.2 gm. of crude fiber and 393.9 gm. of nitrogen-free extract would have been digested, but actually only 28.7 gm. of crude fiber and 371.2 gm. of nitrogen-free extract were digested. There would have been 2159 Cals. of energy digested on the same assumptions and actually 1949 Cals. were digested. Furthermore, if it could be assumed that all of the sugar had been absorbed, as would be reasonable in the case of non-ruminants, and if the basal fraction of the sugar ration had had the same metabolizable energy content that it did when fed alone, it

may be calculated that the metabolizable energy content of the sugar ration would have been 2822 Cals. per kilogram of dry matter instead of 2339. Also the apparent digestibility of the total carbohydrates would have been 78.7 (433.3 ÷ 550.7 × 100) instead of the actual value of 72.6%.

While the metabolizable energy content of the sugar ration was slightly, though definitely, less than that of the basal ration, the metabolizability of the gross energy of the two rations was almost identical, i.e., 54.8 and 54.2% of the gross energy of the basal and of the sugar rations, respectively, were metabolizable.

The indications in this investigation that glucose is readily fermented by the microorganisms of the paunch, are in harmony with the recent in vitro studies of Woodman and Evans ('38) and the in vivo studies of Nikitin ('39).

## SUMMARY

Using six sheep as experimental animals and the double reversal method of feeding, the effect of corn sugar upon the digestibility of the nutrients of a ration was studied with the following results:

1. It increased the apparent digestibility of (a) dry matter, (b) N-free extract, and (c) total carbohydrates.
2. It decreased the apparent digestibility of (a) total nitrogen and (b) crude fiber; and decreased the metabolizable energy content.
3. It had no significant effect upon the apparent digestibility of either ether extract or gross energy.
4. The metabolizability of the energy of the sugar-free ration is approximately the same as that of the sugar-containing ration.
5. It had no effect upon the true digestibility of total nitrogen, since the apparent decrease in digestibility can be entirely accounted for by the estimated increase in metabolic nitrogen in the feces of the sheep on the sugar ration.
6. The rather marked decrease in the digestibility of the fiber when sugar was present was apparently due to the preference by the microorganisms of the paunch for sugar.

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# EFFECT OF CONTROLLED ASCORBIC ACID INGESTION UPON URINARY EXCRETION AND PLASMA CONCENTRATION OF ASCORBIC ACID IN NORMAL ADULTS

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ONE FIGURE

(Received for publication July 28, 1941)

Inasmuch as the optimal intake of ascorbic acid has not yet been satisfactorily determined, it seemed desirable to make a further study of certain criteria for determining requirement, namely, urinary excretion of ascorbic acid and the concentration of ascorbic acid in blood plasma in relation to tissue saturation.

Few studies have been reported in which urinary excretion of ascorbic acid and the concentration of ascorbic acid in the blood have been determined in subjects on a controlled intake of vitamin C over long periods of time. In most cases these studies have been on subjects receiving diets deficient in vitamin C, and blood determinations have not been made daily (Van Eekelen, '36; Rietschel and Mensching, '39; Rietschel and Schick, '39; and Crandon, Lund and Dill, '40).

The study which is most nearly comparable with the one to be reported here is that of Ralli, Friedman and Sherry ('39). In general these investigators proceeded from lower to higher levels of intake as the study progressed. There were four

[1] The data in this paper are taken from the thesis presented by Clara A. Storvick to the faculty of the Graduate School of Cornell University in fulfillment of the requirement for the Ph.D. degree, June, 1941. Further details will be found in the thesis.

instances only in which a period on decreased intake followed a period in which the subjects' tissues were presumably saturated.

In the study which is reported here, the subjects were normal adults who were previously prepared by saturation of the tissues. They were given a basal diet which was low in vitamin C and supplemented, for each experimental period, with a constant level of synthetic ascorbic acid.[2] Urinary excretion and fasting plasma ascorbic acid values were determined daily. Inasmuch as the use of a test dose has proved valuable in indicating the state of tissue reserves, each period was terminated by a standard test dose. The data on the daily urinary excretion and plasma ascorbic acid were treated statistically to determine whether or not there was any correlation between these two values.

## EXPERIMENTAL PROCEDURE

### *Experimental subjects*

Two men and four women served as subjects in this study. They were all actively engaged in some type of nutrition research and were interested in and able to cooperate in every phase of the study. Physical examinations by physicians of the Medical Clinic at Cornell University indicated that they were in normal health. Data as to the sex, age, height, average weight, and the weight variation of the subjects during the course of the experiments are shown in table 1.

### *Methods*

The experimental diet and the methods for the collection and preservation of urine and for the determination of ascorbic acid therein were the same as those reported previously (Belser, Hauck and Storvick, '39). The direct titration with indophenol does not rule out the effect of non-specific reducing substances, but the error due to this circumstance is probably small (Abbasy et al., '35; Belser, Hauck and Storvick, '39).

[2] Hoffman — LaRoche.

Since the levels of ascorbic acid used in this study were relatively high and the urinary excretions correspondingly high, it is improbable that the errors inherent in the method used are of sufficient significance to affect the reliability of the data reported.

Reduced ascorbic acid in plasma was determined by the micromethod of Farmer and Abt ('36) on fasting blood samples. If hemolysis or clotting occurred it was necessary to take a second sample of blood. In some cases this was done

TABLE 1

*Sex, age, height, average weight, and weight variation of the experimental subjects.*

| SUBJECT | SEX | AGE [1] | HEIGHT | AVERAGE WEIGHT | WEIGHT VARIATION | PERIOD OF TIME [2] |
|---|---|---|---|---|---|---|
| | | | *inches* | *pounds* | *pounds* | |
| C.S. | Female | 34 | 68½ | 149 | 19 | Nov. 1939–Feb. 1941 |
| K.J. | Female | 24 | 61 | 108 | 5 | Nov. 1939–Apr. 1940 |
| H.L. | Male | 25 | 71 | 164 | 9 | Feb. 1940–Feb. 1941 |
| E.P. | Male | 31 | 66½ | 116 | 5 | Nov. 1939–Apr. 1940 |
| H.H. | Female | 40 | 62 | 131 | 7 | Nov. 1939–Feb. 1941 |
| J.S. | Female | 22 | 66½ | 139 | 7 | Sept. 1940–Feb. 1941 |

[1] Age at completion of experiment.

[2] The experiments reported in this study come within the periods stated.

after the subject had started to eat his breakfast, but before he had taken the ascorbic acid supplement. Determinations were completed as quickly as possible; the entire time spent from the taking of the first blood sample to the titration of the last sample was about 45 to 60 minutes for five determinations done in duplicate. For 230 plasma ascorbic acid determinations the average difference between duplicates was 0.020 mg. per cent. The differences ranged from 0.000 to 0.042 mg. per cent.

### *Plan of the experiment*

The subjects were prepared by saturation of the tissues with vitamin C during a 3-day pre-experimental period (Belser, Hauck and Storvick, '39). They were considered

saturated if they excreted 200 mg. or more of ascorbic acid in 24 hours.

The experimental periods were 28 days in length. Urinary excretion and fasting plasma ascorbic acid values were determined daily. The various levels of ascorbic acid intake [3] on which the subjects were studied are shown in table 2. The ascorbic acid supplement was taken at breakfast. A test dose of 400 mg. of ascorbic acid, in two 200 mg. doses, one at breakfast and the other at luncheon, was given to each subject on the last day of each experiment. At the close of this 24-hour period, a fasting blood sample was drawn and the ascorbic acid content of the plasma determined to obtain the effect of the test dose on the plasma level of ascorbic acid.

## RESULTS AND DISCUSSION

### *Urinary excretion of ascorbic acid*

The results obtained during twenty-one 28-day periods on various levels of ascorbic acid intake are recorded in table 2. The mean excretion of ascorbic acid was generally much higher for the first than for the second to fourth weeks due to the influence of the generous intake of ascorbic acid during the pre-experimental period. Most of the adjustment of excretion to the new level of intake took place within the first 2 or 3 days. However, even after this adjustment, the urinary excretion was by no means constant. In general the variations were greatest on the highest levels of ascorbic acid intake. This may be seen most easily by examining the 25-day means and their calculated standard deviations (table 2). A similar variation was noted by Ralli and associates ('39).

The mean daily urinary ascorbic acid excretions differed for the various subjects. On the 200 mg. level of ascorbic acid intake, the women (C.S., K.J., H.H., and J.S.) showed higher mean urinary excretion values for ascorbic acid, ranging from 154 to 164 mg., than the men (H.L. and E.P.), whose

[3] In all cases, the ascorbic acid intake refers to the amount of synthetic ascorbic acid taken in addition to approximately 10 mg. which was contained in the experimental diet.

TABLE 2

*The mean,[1] standard deviation, and range of daily urinary excretions of ascorbic acid and fasting plasma ascorbic acid values with coefficient of correlation,[2] and test dose responses for all subjects on various levels of ascorbic acid intake during 28-day experimental periods.*

| LEVEL OF ASCORBIC ACID INTAKE | SUBJECT | URINARY EXCRETION OF ASCORBIC ACID PER 24 HOURS | | | FASTING PLASMA ASCORBIC ACID | | | COEFFICIENT OF CORRELATION [2] | RESPONSE TO TEST DOSE | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | Standard deviation | Range | Mean | Standard deviation | Range | | Ascorbic acid in urine | Ascorbic acid in plasma |
| | | *mg.* | *mg.* | *mg.* | *mg. %* | *mg. %* | *mg. %* | | *mg.* | *mg. %* |
| 200 | C.S. | 154 | 18 | 105–195 | 1.58 | 0.14 | 1.33–1.91 | +0.170 | 261 | 1.53 |
| | K.J. | 164 | 14 | 130–187 | 1.27 | 0.18 | 0.92–1.62 | +0.074 | 289 | 1.12 |
| | H.L. | 135 | 14 | 106–171 | 1.07 | 0.13 | 0.78–1.29 | +0.308 | 261 | 1.12 |
| | E.P. | 130 | 15 | 99–162 | 1.41 | 0.11 | 1.23–1.64 | −0.105 | 219 | 1.60 |
| | H.H. | 163 | 13 | 138–187 | 1.07 | 0.24 | 0.70–1.57 | −0.100 | 267 | 1.05 |
| | J.S. | 158 | 14 | 137–199 | 1.28 | 0.10 | 1.07–1.45 | −0.177 | 290 | 1.51 |
| 150 | E.P. | 89 | 23 | 59–150 | 1.27 | 0.16 | 0.97–1.65 | +0.396 | 221 | 1.51 |
| 125 | C.S. | 82 | 10 | 57– 97 | 1.74 | 0.16 | 1.27–1.99 | +0.191 [3] | 207 | 1.95 |
| | H.L. | 65 | 11 | 40– 89 | 1.05 | 0.15 | 0.69–1.31 | −0.174 | 234 | 1.31 |
| | H.H. | 87 | 11 | 70–111 | 1.05 | 0.09 | 0.85–1.24 | −0.030 [3] | 214 | 1.27 |
| 100 | C.S. | 59 | 10 | 35– 81 | 1.32 | 0.15 | 1.04–1.57 | +0.403 | 250 | 1.71 |
| | H.L. | 47 | 9 | 38– 69 | 1.12 | 0.09 | 0.94–1.30 | +0.306 [3] | 207 | 1.25 |
| | H.H. | 65 | 12 | 47–107 | 0.99 | 0.10 | 0.78–1.14 | −0.453 | 220 | 1.14 |
| 90 | C.S. | 49 | 20 | 23–107 | 1.40 | 0.13 | 1.12–1.68 | −0.065 | 208 | 1.43 |
| | H.H. | 58 | 13 | 42–110 | 0.84 | 0.12 | 0.61–1.17 | +0.226 | 228 | 1.02 |
| 75 | K.J. | 45 | 10 | 30– 66 | 1.17 | 0.10 | 1.00–1.37 | +0.437 | 248 | 1.31 |
| | H.L. | 36 | 10 | 22– 65 | 0.95 | 0.12 | 0.74–1.22 | +0.468 | 76 | 1.17 |
| 65 | K.J. | 38 | 11 | 23– 71 | 1.02 | 0.10 | 0.84–1.32 | +0.463 | 229 | 1.22 |
| 55 | C.S. | 29 | 8 | 19– 52 | 1.24 | 0.18 | 0.92–1.54 | +0.604 | 92 | 1.45 |
| 50 | H.H. | 26 | 11 | 18– 58 | 0.85 | 0.10 | 0.55–1.01 | +0.436 | 121 | 1.03 |
| | J.S. | 26 | 9 | 18– 58 | 1.01 | 0.12 | 0.80–1.23 | +0.599 | 123 | 1.45 |

[1] Mean values were based on 25 of the 28 days, excluding the first 2 days of adjustment to the new level of ascorbic acid intake, and the last day, which represents the response to the test dose.

[2] Values for the coefficient of correlation between the daily urinary excretion of ascorbic acid and fasting plasma ascorbic acid concentration. The significant values of correlation are 0.396 for the 5% level of significance or odds of 19: 1, and 0.505 for the 1% level or odds of 99: 1. The corresponding values are 0.404 and 0.515 when the data for 24 days are used. (Love, H. H., Application of statistical methods to agricultural research, 1937, p. 491.)

[3] Twenty-four day values were used in the calculation of the value of the coefficient of correlation.

mean urinary excretion values were 135 and 130 mg. Similar variations also occurred on the lower levels of intake although there are fewer data available on such levels. There were less marked variations in the mean ascorbic acid excretions in subjects of the same sex (table 2). Data on more subjects are needed before it can be said that men usually excrete less ascorbic acid, on a given level of intake, than women.

Such variations as were noted apparently were not due to differences in urinary pH, since daily determinations using the LaMotte Universal pH outfit, showed only slight variations among the experimental subjects. The median values for the urinary pH on all 28-day periods were as follows: for C.S., 5.6 and for the other subjects, 5.5.

*Fasting plasma ascorbic acid values*

In contrast to the urinary excretion of ascorbic acid the mean fasting plasma ascorbic acid values were characteristic for each individual, and did not indicate any consistent differences between the men and women subjects (table 2). It will be noted that the mean plasma values for the six subjects on the 200 mg. level of intake, when saturation was maintained, varied from 1.07 to 1.58 mg. per cent. It is unlikely that these six subjects include the extremes which might be observed in a large group of normal adults. Goldsmith, Ogaard and Gowe ('41) report higher fasting plasma values for three ambulatory patients for whom these values were determined weekly. Figure 1 shows the daily urinary ascorbic acid excretion and fasting plasma ascorbic acid values for subjects C.S. and H.H. on several levels of intake. Of the six subjects studied, these two had the highest and lowest mean plasma values on a given level of intake. The differences in fasting plasma ascorbic acid values on the same levels of intake were marked, although the mean urinary excretions of ascorbic acid were similar.

Whereas, in general, the standard deviations of the mean urinary ascorbic acid excretions are greater for the higher than for the lower levels of intake, the standard deviations of

the mean fasting plasma ascorbic acid values show no such relationship (table 2). Although in general, higher plasma ascorbic acid values were obtained when the subjects were on the higher levels of ascorbic acid intake and lower values when they were on the lower ascorbic acid intakes, the range of values on each level of intake was so wide that in only one case was the difference between mean plasma values for two periods more than twice the standard deviation of this difference. The one case in which the difference between mean

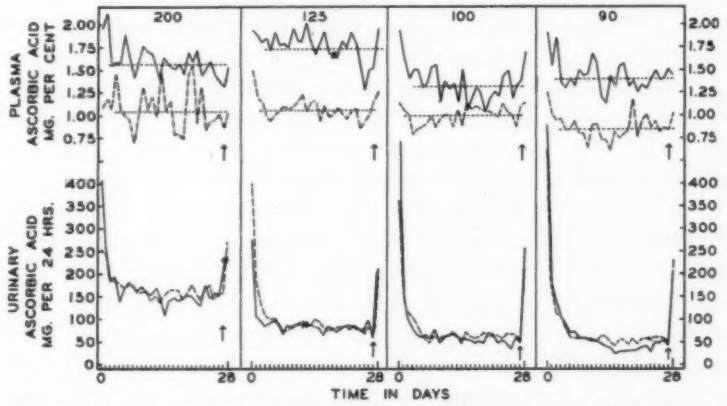


Fig. 1 Daily urinary excretion and fasting plasma ascorbic acid values for two subjects on supplements of 200, 125, 100 and 90 mg. for 28-day periods. Solid lines designate values for subject C.S., dotted lines for subject H.H. Horizontal dotted lines indicate mean fasting plasma ascorbic acid values. An asterisk indicates that a determination is lacking. An arrow indicates a 400 mg. dose of ascorbic acid.

plasma ascorbic acid values on different intakes proved to be statistically significant was when the values for C.S. on the 125 and 55 mg. levels of intake were compared. It is probable that more significant differences would have been observed if the subjects had been studied on lower vitamin C intakes.

The daily variations observed in fasting plasma ascorbic acid values indicate that within the range of intake covered by this study a single determination is of limited value as an index of the state of nutrition with respect to vitamin C. It is true that very high values are likely to indicate adequate

stores of vitamin C and that very low values are usually associated with some degree of depletion of the stores. However, in the present study, a given value for plasma ascorbic acid might be found on widely varying intakes of vitamin C and when the tissue stores were saturated or partially depleted (table 2). For example, values between 1.33 and 1.54 mg. per cent were observed when subject C.S. was receiving supplements of ascorbic acid ranging from 55 to 200 mg. and values between 0.85 and 1.01 were observed when H.H. was receiving ascorbic acid supplements ranging from 50 to 200 mg. This latter subject's mean fasting plasma ascorbic acid value was only 0.84 mg. per cent on a 90 mg. supplement of ascorbic acid, although her output of over 50% of the test dose at the end of the period indicated that she was saturated or nearly so. A similar mean fasting plasma value, 0.85 mg. per cent, was maintained by this subject on a 50 mg. supplement. The response to the test dose following this period indicated partial depletion of the tissue stores (table 2).

As has been suggested by Holmes, Cullen and Nelson ('41), the mean of a series of determinations is probably more useful than individual plasma ascorbic acid values as an index of the state of nutrition with respect to vitamin C. However, a mean plasma value which is indicative of saturation for one subject may signify partial depletion of the stores for another. For example, the highest mean fasting plasma ascorbic acid value exhibited by subject H.H. was 1.07 mg. per cent, when her tissues were saturated on a 200 mg. supplement of ascorbic acid, whereas subject C.S. maintained a mean fasting plasma ascorbic acid value of 1.24 mg. per cent on a daily supplement of 55 mg. of ascorbic acid, which was inadequate to maintain tissue saturation.

### *Response to test dose and estimation of the amount of ascorbic acid necessary to maintain tissue saturation*

The responses to 400 mg. test doses, given at the conclusion of each experimental period, in terms of the urinary excretion of ascorbic acid for 24 hours and the concentration

of ascorbic acid in the plasma on the next morning are shown in table 2.

Although periods of 5 to 7 days may not be sufficient for all subjects to show differences in tissue saturation on low or moderate intakes of ascorbic acid (Belser, Hauck and Storvick, '39), it was believed that they would offer supplementary data on the responses of saturated subjects to the test dose. Data obtained during these short periods are not included in table 2 but each subject was studied during from one to three short periods in addition to a 28-day period on the 200 mg. supplement. This amount of ascorbic acid was considered sufficient to assure tissue saturation.

As has been previously observed (Belser, Hauck and Storvick, '39), considerable variation was noted in responses to the test dose after the several saturation periods. If the lowest response to a test dose after a period on the 200 mg. supplement is used as the criterion of tissue saturation, the following standards are available for these subjects: for C.S., 259; K.J., 249; H.L., 204; E.P., 216; H.H., 267; and J.S., 268 mg. of ascorbic acid. Using these standards, it appears that the following supplements of ascorbic acid were adequate to maintain tissue saturation: for C.S., a little above 100 mg.; K.J., 75 mg.; H.L., 100 mg.; E.P., 150 mg.; and H.H., somewhat above 125 mg. Although E.P. did not receive a supplement less than 150 mg. for a 28-day period, his response to a test dose after receiving 125 mg. of ascorbic acid for 2 weeks was only 187 mg.; hence, he apparently needed more than 125 mg. for tissue saturation. The test dose response given by K.J. after a 2-week period on a 50 mg. supplement of ascorbic acid, was above her standard, but that following the 28-day period on the 65 mg. level of intake was not. There were insufficient data on J.S. to permit the estimation of her requirement for tissue saturation.

The excretion of 50% or more of the test dose within 24 hours has been used commonly as a criterion of saturation (Smith, '38). In this laboratory, no subject has excreted less than 200 mg., that is, 50% of the test dose following a satura-

tion period. If this criterion is used for estimating the smallest supplement of ascorbic acid which maintained tissue saturation for the subjects in this study during the 28-day periods, it appears that the following were sufficient: for C.S., 90 mg.; K.J., 65 mg.; H.L., 100 mg.; E.P., 150 mg.; and H.H., 90 mg. When judged on this basis, test dose responses after 2-week periods, which are not included in the table, indicated tissue saturation for K.J. on a 50 mg. and for H.H. on a 75 mg. supplement, but not for C.S. on a 75 mg. or E.P. on a 125 mg. supplement.

Since all subjects showed a marked urinary response to the test dose on all the levels of intake tested, it may be inferred that none of the levels used was low enough to cause severe depletion of the vitamin C stores during the 28-day period.

In general the fasting plasma ascorbic acid values did not show a rise as a result of the test dose under the conditions of this experiment. In sixteen out of twenty-one cases, the fasting plasma ascorbic acid values following the test dose were within the range for the preceding experimental period. Furthermore, the fasting plasma ascorbic acid values on the morning following the test dose seemed to bear no relationship to the level of ascorbic acid intake for the preceding period. For example, values of 1.45, 1.43, and 1.53 mg. per cent observed in subject C.S. following periods when the supplements were 55, 90, and 200 mg. respectively, are all within the ranges observed on any of the supplements used for this subject.

Whereas Ralli, Friedman and Sherry ('39) concluded that 1.0 mg. per cent represented a normal fasting plasma ascorbic acid value for their subjects during saturation, that an intake of 100 mg. of ascorbic acid was necessary to maintain this level in the plasma, and that higher intakes did not raise the plasma level appreciably, the subjects in this study showed marked individual variation in fasting plasma ascorbic acid values when their tissues were saturated, and they varied markedly in the amounts of ascorbic acid required to maintain this state.

### *Statistical interpretation of the data* [4]

Correlation values [5] were calculated to discover whether or not there was any relationship between the urinary excretion of ascorbic acid and the concentration of ascorbic acid in the plasma when the intake of ascorbic acid was maintained at a constant level. All calculations for the 28-day periods were based on 25-day values which excluded those for the first 2 days of the experimental period, when there was some adjustment to a new level of ascorbic acid intake, and the last day, when the ascorbic acid intake varied from the amount used during the rest of the period. The results are recorded in table 2. For these subjects there was a definite correlation between the daily urinary excretion of ascorbic acid and the concentration of ascorbic acid in the plasma on intakes of 75 mg. and less but no consistently significant correlation between these two variables on higher levels of ascorbic acid intake.

It was also possible to calculate the multiple correlation coefficient when the intake was varied. There were three studies on subject C.S. which were run consecutively. Preceding the 28-day period during which she received 125 mg. of ascorbic acid daily, there were two 14-day periods during which she received no supplement. These periods were separated from each other by several days during which her tissues were resaturated by daily 400 mg. doses of ascorbic acid. Thus there were 65 consecutive days when this subject was on the experimental diet. The value obtained for the multiple correlation coefficient [6] was +0.829 which indicates marked significance, since for the coefficient of correlation for three variables for the degrees of freedom representing 60 days, a value of 0.377 or above is considered highly significant.

[4] The authors are indebted to Dr. H. H. Love and Dr. J. R. Livermore for assistance in the statistical interpretation of the data obtained in these studies.

[5] Croxton, F. E., and Cowden D. J. 1939. Applied general statistics, p. 672. Prentice-Hall Co.

[6] Love, H. H., 1937. Application of statistical methods to agricultural research, pp. 182 and 183. The Commercial Press Limited, Shanghai, China.

Thus there was a high correlation between the urinary excretion of ascorbic acid and the concentration of ascorbic acid in the plasma when there were marked changes in the intake. Inspection of the data indicates that marked increases in urinary excretion of ascorbic acid follow marked increases in intake, but that a similar influence on fasting plasma ascorbic acid occurs only up to the minimum intake required for tissue saturation.

## SUMMARY AND CONCLUSIONS

Urinary excretion of ascorbic acid and fasting plasma ascorbic acid values were determined daily in six normal adult subjects, two men and four women, during twenty-one 28-day periods on controlled intakes of ascorbic acid.

In general, lower urinary and plasma ascorbic acid values were observed on the lower levels of intake but there were marked variations in the same subject from day to day, and between individual subjects.

There was a definite correlation between the daily urinary excretion and the plasma concentration of ascorbic acid on the lower levels but not on the higher levels of ascorbic acid intake.

As estimated from the response to the test dose at the conclusion of each experimental period, supplements of from 65 to 150 mg. of ascorbic acid, in addition to approximately 10 mg. in the basal diet, were required by the subjects in this study to maintain tissue saturation.

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# NUTRITIONAL STUDIES ON POWDERED WOOL[1]

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THREE FIGURES

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It is generally accepted that keratins such as wool are insoluble in ordinary protein solvents and are not digested by the common proteolytic enzymes. Wool that has been treated with alkali at pH 10-11 (Meunier, Comte and Chambard, '27; Fromageot and Porcherel, '31) or with oxidizing and reducing agents (Linderstrøm-Lang and Duspiva, '35; Stary, '28; Waldschmidt-Leitz and Schuckmann, '29; Goddard and Michaelis, '34) is more susceptible to the action of enzymes.

In preliminary studies (Routh and Lewis, '38) it was observed that after wool was ground in a ball mill the powdered material was readily digested by trypsin and pepsin in vitro. These results suggested the possible utilization of powdered wool by the animal organism. This paper is a report of growth studies on rats in which powdered wool was the main source of dietary nitrogen.

## EXPERIMENTAL

The preparation of powdered wool was essentially that described recently in a study of its chemical properties (Routh, '40). Several batches (200–300 gm.) were ground by 500,000 revolutions of the ball mill. The average composition of the

[1] The essential portions of this paper were presented before the thirty-fifth meeting of the American Society of Biological Chemists at Chicago, April 15–19 (1941).

product was 15.70% nitrogen, 3.55% sulfur, 9.03% cystine, and 0.97% ash; the amino nitrogen was 2.23% of the total nitrogen.

Basal diet 15 had the following percentage composition: powdered wool 15, starch 41.4, sucrose 15, agar 2, salt mixture (Hubbell, Mendel and Wakeman, '37) 2.5, hydrogenated cottonseed oil [2] 19, cod liver oil 5, and choline hydrochloride 0.1. Basal diet 20 differed from diet 15 in that it contained 20% powdered wool, 36.4% starch, and 5% cottonseed oil which replaced an equivalent amount of hydrogenated cottonseed oil to overcome the dryness of the diet. As a vitamin supplement each animal received daily 40 μg. riboflavin, 40 μg. thiamine hydrochloride, 200 μg. nicotinic acid and 50 mg. of a rice polish concentrate [3] incorporated in two pills. The pills were fed approximately 12 hours apart. The strain of rats used in this laboratory gained 3–4 gm. per day when 15% casein replaced the powdered wool in the basal diets described above.

Young rats weighing between 37 and 85 gm. were used in these studies. They were housed in individual false-bottomed cages and were fed the basal diets ad libitum. Food consumption and body weights were recorded every 4 days.

Preliminary studies had indicated that animals fed unsupplemented powdered wool as their sole source of protein lost weight less rapidly and lived longer than controls on a nitrogen free diet. To determine which amino acids were needed as supplements to those already present in wool, groups of rats were fed an acid hydrolysate of wool plus various amino acids. In addition to tryptophane which had been destroyed during acid hydrolysis, supplements of both histidine and methionine were found necessary to produce growth. When lysine was also included a slightly better growth was observed.

More extensive studies were then carried out with diets that contained powdered wool supplemented with these amino acids. They were incorporated into the diets at the following

[2] Crisco.

[3] Ryzamin B from Burroughs-Wellcome Co., Tuckahoe, New York; riboflavin and thiamine through the courtesy of Merck and Co.

levels: tryptophane 0.2%, methionine 0.6%, histidine 0.4% (as the monohydrochloride), and lysine 0.6% (as the dihydrochloride). These supplements replaced an equivalent amount of starch in the diet.

Individual growth records are presented in figures 1–3. The curves A to H show the effects of various supplements to the basal diets as outlined in the explanation of the charts.

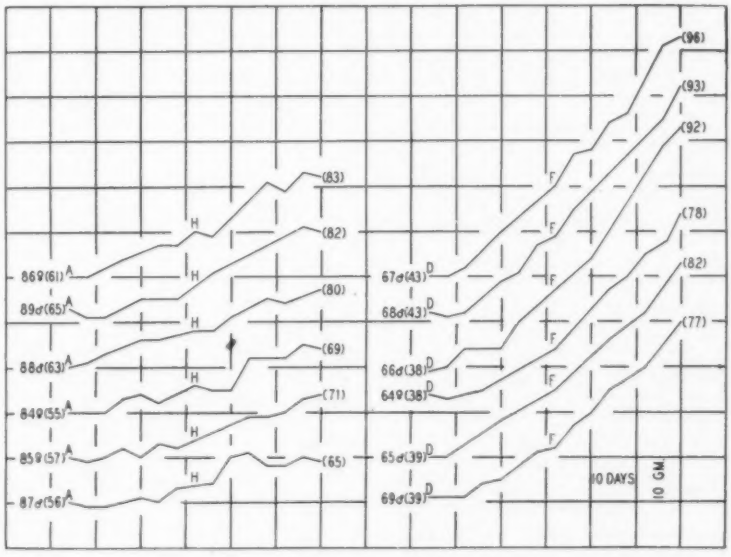


Fig. 1 Initial and final weights are given in parentheses. The various regimes and the food consumption per rat per day were as follows: A — basal diet 15 + 0.6% meth., 0.4% hist., 4.5 gm.; H — basal diet 15 + 0.6% meth., 0.4% hist., 0.6% lysine, 5.0 gm.; D — basal diet 15 + 0.2% trypt., 0.6% meth., 0.4% hist., 4.5 gm.; F — basal diet 20 + 0.2% trypt., 0.6% meth., 0.4% hist., 5.0 gm.

Supplements of tryptophane, methionine and histidine (diet D, figs. 1 and 3) produced moderate growth. When the tryptophane was removed from this diet little more than maintenance resulted (A, fig. 1); addition of lysine (H, fig. 1) did not appreciably increase the rate of growth.

Omission of histidine from diet D caused loss of weight followed by slow growth (B, fig. 2). A somewhat similar

response was observed when methionine was omitted (C, fig. 2).

Growth on diets D and F which contained tryptophane, methionine and histidine was increased by the addition of lysine (E and G). The average gain (fig. 3) in 28 days on diet D was 28 gm., on diet E, 48 gm. The corresponding gain on diet F was 36 gm. and on diet G, 47 gm.

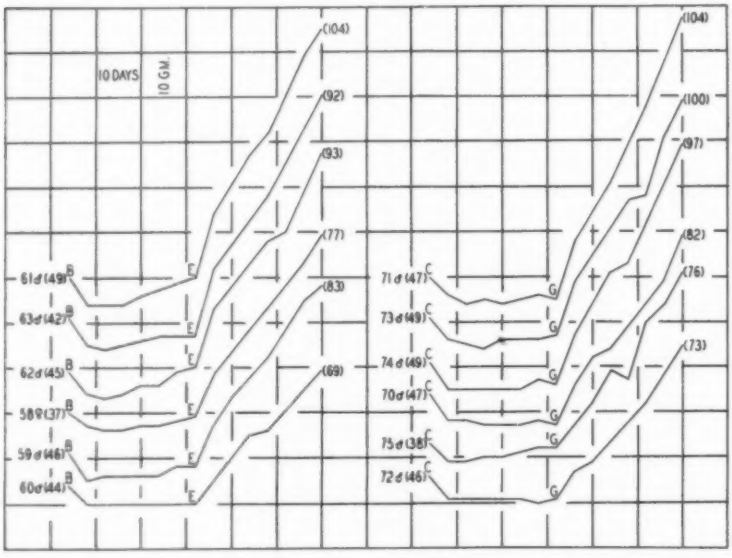


Fig. 2 Initial and final weights are given in parentheses. The various regimes and the food consumption per rat per day were as follows: B — basal diet 15 + 0.2% trypt., 0.6% meth., 2.8 gm.; C — basal diet 15 + 0.2% trypt., 0.4% hist., 2.8 gm.; E — basal diet 15 + 0.2% trypt., 0.6% meth., 0.4% hist., 0.6% lysine, 4.8 gm.; G — basal diet 20 + 0.2% trypt., 0.6% meth., 0.4% hist., 0.6% lysine, 4.7 gm.

A change from basal diet 15 to diet 20 resulted in every case in an increased rate of growth (compare D and F, also E and G, fig. 3). This was probably due to the increased amount of amino acids available.

The average food consumption for the rats on each diet is included in the explanation of the charts. A striking increase

in food consumption was noted when a diet that failed to support growth was replaced by one that produced a moderate growth (compare B and C to E and G). The increase was not as marked when the rats were changed from a diet that produced fair growth to one that resulted in a moderate growth (compare A and D with H and F).

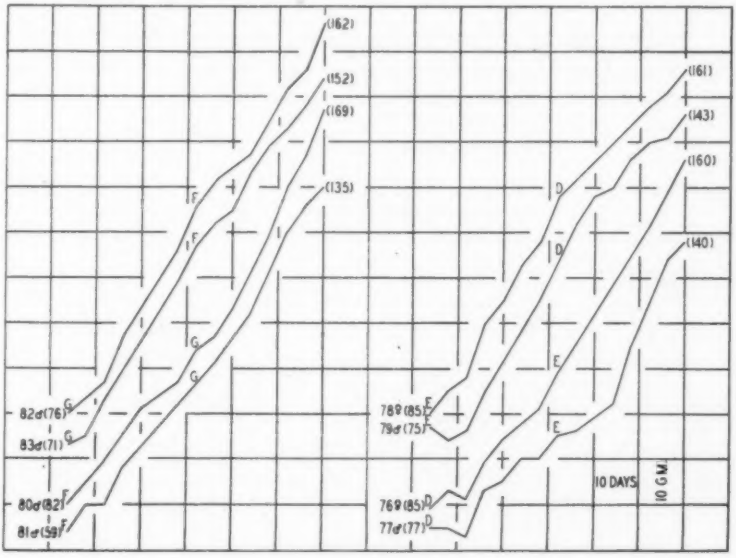


Fig. 3 Initial and final weights are given in parentheses. The various regimes and the food consumption per rat per day were as follows: D — basal diet 15 + 0.2% trypt., 0.6% meth., 0.4% hist., 7.0 gm.; E — basal diet 15 + 0.2% trypt., 0.6% meth., 0.4% hist., 0.6% lysine, 6.7 gm.; F — basal diet 20 + 0.2% trypt., 0.6% meth., 0.4% hist., 6.6 gm.; G — basal diet 20 + 0.2% trypt., 0.6% meth., 0.4% hist., 0.6% lysine, 6.4 gm.

The rate of growth on diet 15 supplemented with tryptophane, methionine, histidine and lysine was slightly greater than that obtained on the wool hydrolysate supplemented with the same amino acids. The average gain on basal diet 15 was 1.5 gm./day as compared to 1.2 gm./day on the wool hydrolysate diet. This suggests that the powdered wool is assimilated quite as readily as the hydrolyzed wool. More

extensive studies on the digestibility and utilization of powdered keratins are being carried out.

## CONCLUSIONS

Powdered wool as the sole source of protein in an otherwise adequate diet is incapable of supporting growth in young rats. Tryptophane, methionine, histidine, and lysine were found to be present in suboptimal concentration in the powdered keratin. The supplementation of the basal diet with these amino acids resulted in moderate growth.

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# THE INTERRELATIONSHIP OF MANGANESE, PHOSPHATASE AND VITAMIN D IN BONE DEVELOPMENT

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Robison ('23) reported that the phosphatase activity of the bones of rachitic rats is greater than that of the bones of normal rats. Kay ('30) found that an increased phosphatase content of the blood plasma accompanies rickets and certain other generalized bone diseases of human beings such as osteomalacia, osteitis deformans and generalized osteitis fibrosa. Wilkins and Regen ('35) reported that in bone fractures there is a rapid rise in the phosphatase activity at the site of the injury. Auchinachie and Emslie ('33) found that the phosphatase activity of the blood plasma of sheep, fed a rachitogenic diet, is increased three to four times its normal value. On the other hand, Wiese and associates ('39) reported that the activity of the phosphatase of blood plasma and bone is lowered in perosis caused by manganese deficiency. This is a disease of chicks characterized by malformation of the bones. Caskey, Gallup and Norris ('39) presented evidence showing that the ash content of the bones of chicks afflicted with perosis is slightly lower than that of the bones of normal chicks.

Since the phosphatase activity of the blood plasma and bone is greatly increased in rickets and certain other bone diseases of man and other species, and since the activity is markedly reduced in manganese deficiency in chicks, it was thought that, if manganese functions in bone formation by playing an active part in the production of bone phosphatase, this role could

be more clearly demonstrated by observing the effect of manganese deficiency upon bone phosphatase activity and calcification in chicks fed rachitogenic and non-rachitogenic diets. Therefore, studies were conducted in an effort to obtain by this procedure a clearer conception of the function of manganese in bone formation, the results of which are presented in this report.

## EXPERIMENTAL PROCEDURE

Two experiments were conducted in this work. Rhode Island Red cockerels were used as the experimental animals. The chicks were divided into groups approximately 24 hours after hatching, weighed individually and confined in metal cages. They were weighed at weekly intervals thereafter and any symptoms of perosis scored as to severity according to the procedure of Wilgus, Norris and Heuser ('37). Feed and water were always available to the chicks. The duration of each experiment was 8 weeks.

The basal diets (A and B) used in these experiments are given in table 1. These rations contained approximately 20% protein. Basal diet A fed in experiment 1 was found by

TABLE 1

*Basal diets*

| INGREDIENTS | DIET A, EXPERIMENT 1 | DIET B, EXPERIMENT 2 |
|---|---|---|
| | % | % |
| Degerminated yellow corn meal | 64.00 | 64.00 |
| Dried skim milk | 10.00 | 15.00 |
| Meat scrap (55% protein) | 10.00 | 5.00 |
| Dried brewers' yeast | 5.00 | 5.00 |
| Liver meal | 5.00 | 6.50 |
| Soybean oil | 2.88 | 2.88 |
| Calcium carbonate | 1.50 | 1.00 |
| Steamed bone meal | 1.00 | —— |
| Iodized salt | 0.50 | 0.50 |
| Shark liver oil (2500 I.U. of vitamin A per gram) | 0.12 | 0.12 |
| | 100.00 | 100.00 |

analysis to contain 2.9 mg. of manganese per 100 gm., and basal diet B fed in experiment 2 was found to contain 2.8 mg. per 100 gm. The amounts of calcium and phosphorus in basal diet A were 1.7% and 0.9% respectively, while those in basal diet B were 1.1% and 0.7% respectively. Both diets produced uniform perosis, but diet B produced the more severe rickets.

Manganous sulfate, c.p., was used as the source of supplementary manganese in these studies and cod liver oil containing 250 I.U. of vitamin D per gram was used to supply supplementary vitamin D.

*Experiment 1.* In this experiment, two groups of forty chicks each and two groups of twenty chicks each were employed. One group of forty chicks (negative control) received basal diet A only. A second group of twenty chicks received the basal diet plus 5 mg. of manganese per 100 gm. A third group of forty chicks received the basal diet plus 0.5% of cod liver oil. The last group of twenty chicks (positive control) received the basal diet plus 0.5% of cod liver oil and 5 mg. of manganese per 100 gm.

At the end of 4 weeks, eight chicks representative as to body weight and perotic condition were selected from each group and killed, after which the tibiae were removed for analysis. At this time the remaining chicks in the group receiving the diet containing no supplementary manganese and those in the group receiving the diet containing neither supplementary manganese nor vitamin D were each divided into two equal lots. One lot of each group was fed the same diet as previously. The other lot was given its previous diet supplemented with 5 mg. of manganese per 100 gm. At the end of 8 weeks, all the remaining chicks were killed and the tibiae removed for analysis.

The left tibia of each chick was used in determining the moisture, fat and ash content, while the right tibia was used in determining bone phosphatase activity. The right tibia was removed as soon as possible after killing the chick, weighed and cut into small pieces. The pieces of bone were then placed in an Erlenmeyer flask and made to such a volume with dis-

tilled water that the suspension contained 5% of bone. Five drops of chloroform were added, the flask tightly stoppered and the tissue extracted for 60 hours at room temperature. The extract was then filtered and the phosphatase activity of the filtrate determined by the method used by Wiese and associates ('39).

*Experiment 2.* In this experiment, four groups of twenty-five chicks each were used: One group (negative control) received basal diet B only; a second group received this basal diet plus 7.5 mg. of manganese per 100 gm.; a third received the basal diet plus 0.5% of cod liver oil; the last (positive control) received the basal diet plus 0.5% of cod liver oil and 7.5 mg. of manganese per 100 gm.

Representative chicks were taken from each group at the end of the fourth, sixth and eighth weeks, and analyses were made as in the first experiment. In this experiment, however, the right femur was used for the determination of bone phosphatase activity and the left tibia for the determination of moisture, fat and ash content, while the right tibia was sectioned longitudinally and stained by the silver nitrate procedure in order to determine whether or not rickets was present.

## RESULTS AND DISCUSSION

The results for the bone analyses of experiments 1 and 2 are given in tables 2 and 3, respectively. The bone phosphatase activity of the chicks, fed an adequate diet except for a lack of manganese, was markedly reduced as compared to that of chicks fed the same diet supplemented with manganese (positive controls). This was true for all ages of chicks in both experiments. The results confirm those obtained previously by Wiese and associates ('39).

When the chicks received a rachitogenic diet containing an adequate amount of manganese, the bone phosphatase activity was increased to almost twice the amount found in normal chicks (positive controls) of the same age. These results show that in rachitic chicks the bone phosphatase mechanism reacts

in the same manner as that reported for the rat by Robison ('23).

The phosphatase content of the bones, when manganese was omitted from the rachitogenic diet, remained at approximately the normal level instead of increasing to the abnormally high level of the bones of chicks fed a typical rachitogenic diet. The bone phosphatase activity of chicks, which received no supplementary manganese during the first 4 weeks but received

TABLE 2

*Results of experiment 1*

| TREATMENT | CHICKS | WEIGHT | FEED PER CHICK PER PERIOD | RICKETS PRESENT | PEROSIS INDEX | ASH IN FAT-FREE DRY BONE | PHOSPHATASE ACTIVITY |
|---|---|---|---|---|---|---|---|
| | *no.* | *gm.* | *gm.* | | | *%* | *units* [1] |
| | | | 4 weeks | | | | |
| —Mn —D | 8 | 226 | 429 | Yes | 37.5 | 36.6 | 7.19 |
| +Mn —D | 8 | 313 | 633 | Yes | 7.3 | 36.8 | 15.24 |
| —Mn +D | 8 | 234 | 402 | No | 42.8 | 45.2 | 5.02 |
| +Mn +D | 8 | 326 | 593 | No | 1.2 | 45.8 | 7.71 |
| | | | 8 weeks | | | | |
| —Mn —D | 11 | 376 | 713 | Yes | 54.6 | 36.0 | 5.82 |
| +Mn —D [2] | 11 | 589 | 932 | Yes | 27.8 | 37.3 | 10.21 |
| +Mn —D | 11 | 663 | 1101 | Yes | 7.1 | 39.1 | 12.70 |
| —Mn +D | 13 | 521 | 1013 | No | 51.7 | 43.3 | 3.14 |
| +Mn +D [2] | 14 | 685 | 1168 | No | 14.1 | 44.8 | 4.55 |
| +Mn +D | 11 | 868 | 1391 | No | 0.0 | 45.9 | 5.01 |

[1] One phosphatase unit is the amount of enzyme which, when allowed to act upon an excess of disodium phenyl phosphate at pH 9.0 for 30 minutes at 37.5° C., will liberate 1 mg. of phenol.

[2] These groups received no manganese during the first 4 weeks but received 5 mg. of manganese per 100 gm. of diet during the last 4 weeks.

manganese at the rate of 5 mg. per 100 gm. of diet for the last 4 weeks, was intermediate. It is evident from these results that manganese is essential for the abnormally great accumulation of phosphatase in the bones of rachitic chicks as well as for normal bone of chicks. Moreover, it appears possible that manganese is also essential for this function in other species of animals, since in "uncomplicated" rickets in chicks the phosphatase content of the bone increases greatly, the same as in the rat.

The difference in the phosphatase level of the bones of groups of chicks fed rachitogenic diets, with and without an adequate amount of manganese, was much greater than that obtained in the bones of groups of chicks fed non-rachitogenic diets, with and without an adequate amount of this element. The greatly increased difference in bone phosphatase level obtained in these studies by the use of rachitogenic diets provides a more specific procedure for determining the effect of manganese upon the level of bone phosphatase than has been used previously.

TABLE 3

*Results of experiment 2*

| TREATMENT | CHICKS | WEIGHT | FEED PER CHICK PER PERIOD | RICKETS PRESENT | PEROSIS INDEX | ASH IN FAT-FREE DRY BONE | PHOSPHATASE ACTIVITY |
|---|---|---|---|---|---|---|---|
| | *no.* | *gm.* | *gm.* | | | *%* | *units* |
| | | | 4 weeks | | | | |
| —Mn —D | 6 | 216 | 460 | Yes | 18.4 | 33.9 | 7.70 |
| +Mn —D | 6 | 226 | 450 | Yes | 0.2 | 37.8 | 12.20 |
| —Mn +D | 6 | 251 | 444 | No | 27.6 | 42.4 | 4.02 |
| +Mn +D | 6 | 262 | 521 | No | 0.2 | 43.6 | 6.58 |
| | | | 6 weeks | | | | |
| —Mn —D | 8 | 352 | 400 | Yes | 32.3 | 34.6 | 7.72 |
| +Mn —D | 8 | 391 | 451 | Yes | 1.5 | 37.1 | 13.06 |
| —Mn +D | 8 | 421 | 485 | No | 31.5 | 43.9 | 2.57 |
| +Mn +D | 8 | 500 | 606 | No | 0.0 | 45.5 | 6.03 |
| | | | 8 weeks | | | | |
| —Mn —D | 9 | 473 | 555 | Yes | 40.7 | 36.8 | 8.87 |
| +Mn —D | 9 | 529 | 542 | Yes | 0.5 | 38.3 | 14.53 |
| —Mn +D | 9 | 603 | 604 | No | 27.6 | 43.4 | 3.06 |
| +Mn +D | 9 | 751 | 805 | No | 0.0 | 44.8 | 6.71 |

In experiment 2 (table 3) the ash content of the bones of chicks fed an adequate diet except for a deficiency of manganese was lower than that of chicks fed the same diet supplemented with 7.5 mg. of manganese per 100 gm. Also, the ash content of the bones of chicks fed a rachitogenic diet deficient in manganese was lower than that of chicks fed the same diet supplemented with 7.5 mg. of manganese per 100 gm. In experiment 1, the same was true at the end of the 8-week period, but

no differences were found at 4 weeks of age. The bone ash reduction was biologically significant and not due to differences in chick weight, as the bone ash of the 8-week-old chicks, fed a low-manganese diet, was less than that of chicks of a younger age and lower weight fed a diet adequate in manganese. These results are in agreement with those previously reported by Caskey, Gallup and Norris ('39) but not with the conclusions of Wiese and associates ('40) who believed that the slightly greater quantity of calcium and phosphorus in various portions of the tibiae of normal chicks as compared to perotic ones was not significant.

The perosis index was observed to be correlated inversely both with the bone ash and the phosphatase activity. It was also found that, after 4 weeks of age when the chicks showed symptoms of rachitic lameness, the perosis indices of the rachitic lots of chicks were somewhat greater than those of the non-rachitic lots. This is believed to be due to the fact that enlargement of the distal ends of the tibiae and the proximal ends of the tarso-metatarsi occurs in rickets as well as in perosis and cannot be differentiated in arriving at the perosis index. If this is true, then the development of rickets slightly retards the onset of perosis, since the perosis indices at the end of the first 4 weeks of the lots of chicks which did not receive either vitamin D or manganese were less than those of the corresponding lots which received vitamin D.

In experiment 1 the fat content of the bones of the chicks receiving an adequate quantity of manganese was greater than that of the bones of chicks fed a diet deficient in manganese. No important differences in fat content were obtained, on the other hand, in experiment 2. Whether or not this discrepancy in results is related to the differences in the calcium and phosphorus content of the basal diets or to some other causes cannot be determined from the evidence available.

Upon examination of the longitudinal sections of the tibiae after staining with silver nitrate, it was observed that the metaphyses of the tibiae of the chicks receiving an adequate diet except for a deficiency of manganese were only approxi-

mately one-half as wide as the metaphyses of the tibiae of the comparable normally fed chicks. Furthermore, the epiphyses of the tibiae of the chicks fed the manganese-deficient diet were very weakly united to the diaphyses. Upon partial dehydration the epiphyses almost invariably separated automatically from the diaphyses in the stained longitudinal sections of the non-rachitic tibiae of chicks fed a low-manganese diet. This did not occur in the normal tibiae. Titus ('32) has reported that the union of the epiphysis and the diaphysis is delayed in chicks afflicted with perosis.

The lowering of the bone phosphatase level in "uncomplicated" manganese deficiency and in manganese deficiency complicated with rickets shows that there is an intimate relationship between the phosphatase activity of the bones and the manganese content of the diet. The results suggest, moreover, that in manganese deficiency the lowering of the phosphatase level retards bone development with the result that the bones are shortened, the ash content of the bones is reduced and the strength of the union between the epiphysis and the diaphysis is greatly reduced. In view of the results of Caskey, Gallup and Norris ('39) it is probable that the deficiency of manganese in the diet causes a disproportionately greater retardation in the development of bones in chicks during growth than of other parts of the body with the result that the bones are not strong enough to support the weight of the body and malformation occurs. That such malformation is not limited only to the chick is suggested by the results of Miller, Keith, McCarty and Thorp ('40) with swine and by some observations of Barnes, Sperling and Maynard ('41) with the rat. Also, a comparison of the symptoms of perosis with those of "slipped epiphyses" in children, recently reviewed by Ghormley and Fairchild ('40), indicates a possible relationship between these bone malformations.

## SUMMARY

In rachitic chicks it was found that the bone phosphatase is increased to an abnormally high level the same as in the

rachitic rat. By omitting manganese from the rachitogenic diet, the bone phosphatase was decreased to approximately the normal amount. The difference in the phosphatase levels in the bones of groups of chicks fed rachitogenic diets, with and without an adequate quantity of manganese, was found to be much greater than the difference in the phosphatase levels in the bones of groups of chicks fed a non-rachitogenic diet, with and without an adequate quantity of manganese.

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# FETAL RAT PARATHYROIDS AS AFFECTED BY CHANGES IN MATERNAL SERUM CALCIUM AND PHOSPHORUS THROUGH PARATHYROIDECTOMY AND DIETARY CONTROL

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The general question of fetal autonomy under experimental modification of maternal serum components is a nearly virgin field for research. As an extension of our published work on the effect of pregnancy and dietary influence on the size of the parathyroid gland of albino rats we now consider the effect of these factors on the fetuses of the females previously studied. The work is the morphological aspect of a series of experiments done in collaboration with M. Bodansky and V. Duff.[1] The present paper will compare the effect of synthetic diets when supplied to pregnant rats (1) in relation to their parathyroids and those of their progeny, and (2) the effects of the same diets supplied to parathyroidectomized rats and their progeny.

The diets are from the list of Cox and Imboden ('36). Bodansky and Duff ('41 a), who discuss these diets critically, also tabulate the changes in serum calcium and phosphorus and calculate a coefficient of reproductive success. The coefficient compares dietary effects on average pup weights and litter size in normal and parathyroidectomized animals and is useful in establishing the normal diet in our work on gland size.

Adult parathyroids were removed by operation prior to pregnancy or were excised from the larynges of animals sacri-

[1] The colony was reared and chemical determinations were made in the John Sealy Memorial Laboratory, Galveston, Texas.

ficed at the termination of pregnancy. Newborn pups were sorted by weight, sacrificed, the body cavities opened and the entire body then fixed in 10% formalin plus 1% formic acid. Within about 24 hours each larynx was removed and again fixed for several hours before dehydration with dioxan and infiltration with paraffin. The fetal larynx with the thyroid in situ was cut serially. Methods of weighing from paper sections were the same as for adult parathyroids (Sinclair, '41). We tabulate the weights of single parathyroids.

### NORMAL DIET AND REFERENCE VALUES OF PARATHYROID WEIGHTS

Diet 7 was found by Cox and Imboden as well as by Bodansky and Duff to be satisfactory for reproduction. We originally considered it an optimum diet, but if enlargement of the parathyroid gland is any indication of dietary deficiency, diet 7 is not adequate during pregnancy. The adult parathyroid on this diet enlarges from 86 μg. in the virgin state to 114 μg. during the first pregnancy, to 208 μg. during the second pregnancy and does not regress later. However, since this ration seems to be excellent in respect to other aspects of reproduction such as litter size and weight we will consider it the normal for comparison.

The normal fetal parathyroid weight in a large series is 7.7 μg. when the pregnant rat is fed diet 7. Meigs et al. ('19) stated that the effects of deficient diets are not apparent until after several litters have been cast. We find no change either in gland weight or in pup size with successive litters. Variation is not excessive. Gland weights in pups of respective body weights averaged as follows: less than 4 gm., 5.8 μg.; 4.1–5.0 gm., 6.0 μg.; 5.1–5.5 gm., 6.6 μg.; 5.6–6.0 gm., 7.7 μg.; 6.1–6.5 gm., 8.5 μg.; 6.6 gm. and over, 9.8 μg. There are no sex differences. Right-left asymmetry is small and favors neither side. The self-regulating mechanism of the fetus is shown to advantage by comparing the effect of diet 7 with 7 v in which 100 to 400 units of viosterol were added daily. We treated the viosterol fed animals as one group. Serum calcium varied both above and below normal. Gland enlargement was kept at 117 μg. instead of the average 141 for diet 7. Fetal para-

TABLE 1

*Fetal parathyroid variation with diet and parathyroidectomy.*

| DIETS | | | BEFORE PARATHYROIDECTOMY | | | | | | | AFTER PARATHYROIDECTOMY | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Ca % | P % | Mater. serum Ca mg. | Mater. serum P mg. | M. para μg. | Fetal wt. gm. | F. para μg. | Stand. dev. | No. | Mater. serum Ca mg. | Mater. serum P mg. | Fetal wt. gm. | F. para μg. | Stand. dev. | No. |
| 7 | 0.49 | 0.49 | 9.10 | 4.67 | 141 | 5.45 | 7.7 | 2.7 | 316 | 4.65 | 7.81 | 3.49 | 8.8 | 2.8 | 113 |
| 8 | 0.49 | 0.735 | 8.80 | 6.07 | 179 | 4.95 | 5.6 | 1.5 | 13 | 3.40 | 8.00 | 5.58 | 9.3 | — | 2 |
| 10 | 0.49 | 2.450 | 9.88 | 4.97 | 158 | 4.88 | 7.0 | 1.5 | 11 | 3.20 | 5.25 | 2.26 | 11.7 | 3.1 | 4 |
| 12 | 0.735 | 0.495 | 9.36 | 5.66 | small | 5.69 | 8.0 | 3.0 | 11 | 4.50 | 7.39 | 5.43 | 8.0 | 3.0 | 17 |
| 16 | 1.125 | 0.245 | 11.77 | 2.50 | 124 | 5.24 | 5.6 | 1.5 | 67 | 11.50 | 1.90 | 4.62 | 6.0 | 1.7 | 68 |
| 19 | 1.125 | 1.225 | 8.09 | 5.26 | 197 | 5.19 | 8.8 | 1.8 | 84 | 3.85 | 8.34 | 3.50 | 8.8 | 1.9 | 44 |
| 26 | 0.017 | 0.245 | 8.18 | 3.90 | 265 | 5.14 | 7.7 | 2.4 | 117 | 4.06 | 5.44 | 5.14 | 11.0 | 2.8 | 41 |
| 27 | 0.122 | 0.245 | 9.14 | 5.13 | 191 | 4.95 | 5.3 | 1.7 | 29 | 4.24 | 10.76 | 3.67 | 10.2 | 2.7 | 22 |
| 7 v | + 100 I.U. | | 9.15 | 5.13 | 117 | 5.4 | 7.7 | 1.8 | 106 | 3.90 | 7.01 | 5.4 | 9.3 | 2.0 | 110 |
| | + 400 I.U. | | 8.82 | 3.14 | | | | | | 4.95 | 6.83 | | | | |
| 27 v | + 100–200 I.U. | | 9.34 | 8.16 | 122 | 5.2 | 6.9 | 1.6 | 20 | 5.0 | 10.71 | 5.5 | 9.3 | 2.4 | 18 |
| 7 al | + 4% al. acetate | | | | | 4.5 | | | | 7.68 | 4.23 | 4.6 | 6.2 | 2.2 | 25 |
| 11 | total no. cases | | | | 296 | | | | 774 | | | | | | 464 |

Diets are from Cox and Imboden ('36). v = 100 to 400 I.U. of viosterol added daily to diets 7 and 27. al = 4% aluminum acetate added to diet 7. Ca and P per 100 cc. for viosterol and aluminum are averages from Bodansky and Duff, yet to be published, while all other serum values are from the work already published. The pups are samples from their stocks and show the same weights. Maternal parathyroid weights are averages from the complete table by Sinclair ('41).

thyroids show no change whatever, growing to the normal 7.7 μg. with less range of variation than on diet 7 unmodified in any way. Body weights were not increased. It seems as if this value may be regarded as a basis for comparison. All fetal calcium storage is calculated from the table of Bodansky and Duff ('41 b), using as the normal their value for storage under diet 7.

### EFFECT OF DIET

The high phosphorus of diet 8 prevents calcium utilization. Serum calcium drops and phosphorus rises and the maternal parathyroid enlarges. Fetuses developed under these conditions average 91% of the size on diet 7 but their parathyroid glands weigh 5.6 μg., which is only 73% normal. Calcium storage is 91% normal.

Under diet 10 phosphorus is eliminated at a very high rate as shown by a serum phosphorus lower than for diet 8. Serum calcium is held at a supernormal level and the parathyroid gland is enlarged 12%. Corresponding to the high maternal serum calcium the gland of the fetus is depressed to 7.0 μg. or 91% normal. The fetuses average 89% normal weight and calcium storage is 90% normal.

Diet 16 is rachitogenic because of its low phosphorus content though it contains a great excess of calcium. Cox and Imboden ('36), keeping pregnant rats on various diets, calculated the ash content of young rats at the end of lactation as a percentage obtained between the lowest and highest group values. For diet 7 this was 93.7 and for diet 16 only 59.2. At birth the fetal ash total is the lowest recorded and calcium storage is 69% normal. According to Bogert and Plass ('23) the fetus maintains a calcium level about 16.2% above that of the mother. This depresses the fetal gland but it does not improve calcium storage.

Diet 27 is low in both calcium and phosphorus, yet the serum levels of both are kept nearly normal. The maternal parathyroid is very large (191 μg.); this depresses the fetal parathyroid. Fetuses are 91% of normal size and their parathyroids are 61% of normal. Addition of viosterol to diet

27 keeps the parathyroid weight below that for diets 7 and 27. Fetuses of this group are nearly normal in body weight, their parathyroids 90% of normal weight, and calcium storage is 94% of normal.

Diet 12 causes a slight shift upward in both serum calcium and phosphorus. Our few data show a small parathyroid. Consistent with it the fetuses from these mothers are heavier by 6%, have enlarged glands and a calcium storage supernormal by 18%.

Diet 19 causes a depression in serum calcium, a slight rise in phosphorus and nearly 30% increase in maternal gland size. Maternal needs prevent any excess hormone reaching the fetus. The fetal size is 5% below and the parathyroid gland 15% above normal. Serum calcium below the critical level (a little above 8 mg./100 cc.) is probably responsible for a drop in fetal calcium and is the efficient parathyroid stimulus. Calcium storage is 91% normal.

Maternal serum does not reflect fully the low calcium content of diet 26 because its low phosphorus permits retention. The parathyroid does reflect it in successive increases with each pregnancy: the first carries it from 86 to 142 μg.; the second carries it to 180, the third to 224, the fifth to 460, the sixth to 743 μg. This enormous gland provides no hormone in excess of maternal needs. Reproduction on this diet is difficult but the progeny, both in number and weight, are nearly normal. This normality extends to the fetal parathyroid which weighs 7.7 μg., like those of diets 7 and 7 v. It extends also to successive litters. Calcium storage at birth is 88% normal when diet 26 has been used throughout and 99% if diet 7 is used during the early part of gestation. In pups at the end of lactation Cox and Imboden calculated the coefficient of calcification (ash content) for diets 26 and 7 to be 92.7 and 93.7 respectively. The maintenance of normal weight of body, glands and bone ash in fetus and suckling rat under such an extreme diet shows remarkable utilization.

We assume that the concentrations of serum calcium and phosphorus found in the rats at term were representative of the serum levels during the effective portion of the cycle of

pregnancy. An enlarged maternal parathyroid is correlated with a subnormal parathyroid under diets 8, 10, 27, and 27 v, using the gland sizes under diet 7 for comparison. Diets 8 and 10 produced opposite effects on maternal serum calcium and phosphorus. The presence of excess parathyroid hormone seems to be of greater weight than the level of calcium if the latter does not reach a critical low value a little above 8.0 mg./100 cc. In order to affect the parathyroid the hormone must necessarily pass the placenta. Since the extract of Collip is a colloidal protein it can hardly be identical with the secretion of the parathyroid gland as it is liberated into the blood stream. While there is some conflicting evidence it seems that molecules of that size do not normally pass the placenta. A necessary stimulus to the growth of the parathyroid gland is the complete utilization of its secretion. Free hormone in the fetal circulation depresses the fetal gland just as parathyroid hormone injection shrinks the adult gland.

Normal pregnancy is a greater strain on the homeostatic mechanism for calcium than is indicated by the measure of serum calcium and phosphorus. It may appear in bone ash measurements after successive pregnancies. The parathyroid gland is a more sensitive indicator and its hypertrophy under what is considered a normal good diet shows that the diet of pregnancy must be specially compounded. Phosphorus interferes very much with full utilization of calcium and must be lowered somewhat while calcium content should be raised. Neither should be altered to an extreme degree because phosphorus is as necessary to normal ossification as is calcium. It is recognized that whole populations may adapt themselves to low calcium intake by improved retention (Nicholls and Nimalasuriya, '39). We do not, however, know the full effect of such adaptation. Shelling ('35) pointed out that tetany in the newborn is associated with maternal hypofunction.

### EFFECTS OF PARATHYROIDECTOMY

Parathyroidectomy removes the compensating gland factor and permits dietary deficiencies to be fully expressed. Table 1 shows that maternal parathyroidectomy has enlarged the

fetal parathyroid under all but two diets. We note also that the range of absolute increase (52%) or decrease (20%) of fetal parathyroids, like that due to diet alone, is not as great as occurs in maternal parathyroids. This is expected since the mother serves as a buffer between her dietary intake and the homeostatic mechanism of the fetus. This does not prevent a very considerable drop in fetal calcium storage.

Bodansky and Duff ('41 a) show that the coefficient of reproductive success for diet 7 in parathyroidectomy is only 0.733. After parathyroidectomy diet 7 can in no way be considered optimum for reproductive success. Under diet 7 maternal calcium falls far below the critical value and the fetal parathyroid enlarges 14% while body weight drops to 64% of normal. Hoskins and Snyder ('27, '33) found that in dogs an injection of parathyroid hormone directly into the fetus raised calcium level by nearly 50% without in any way altering that of the mother. This is not a simple physical equilibrium between fetal and maternal circulations. The fetus can extract calcium from a maternal source reduced to half its value. Under diet 7 calcium storage per fetus falls to 46% normal.

Shelling ('35) showed that viosterol could produce hypercalcemia even after parathyroidectomy and regardless of diet. If so, under diet 7 v the fetal gland should be nearly normal or reduced. It is enlarged 21% and is larger than on diet 7 alone. The average pup weight, however, was increased 40% at the same time. Bodansky and Duff (unpublished data) show a calcium increase with 100 I.U. of viosterol daily and an unexplained decrease with 400 I.U.

On diet 8 the fetal glands enlarge 65% from a level below the normal for diet 7 to one above that of diet 7 combined with parathyroidectomy. The adequate stimulus is low serum calcium. Fetal calcium storage is 72% normal.

In parathyroidectomized mothers on diet 10 the fetal gland enlarged 67% and fetuses were only 41% normal weight. Fetal calcium storage is 23%, the lowest for any diet used. The number of cases is too few to be decisive but when one

considers the low values of maternal serum calcium and phosphorus the data are in line with expectation.

A large series of animals were raised on diet 16. The combination of excess calcium and low phosphorus permitted a high level of serum calcium in the parathyroidectomized rat. A potentially small gland was removed and the fetal gland failed to enlarge appreciably. The coefficient of reproduction for this diet, 0.913, is the best for any diet considered but it does not reflect the low ash content of the fetus (69% normal) and suckling rat (Cox and Imboden, '36). Storage with this diet is better than with diet 7 and is nearly as high as with diets 8 and 12.

Diets 16 and 7 al gave similar results. Under diet 16 hypercalcemia of the mother seems very reasonable and the suppressive effects on the fetal parathyroid are expected. The mechanism of the effect in 7 al is less obvious. Jones ('36) obtained the same calcium retention and phosphorus lowering after feeding aluminum salts with normal and deficient rations but offered no explanation of the phenomenon. The mechanism seems to be in the intestine. Aluminum salts are generally not readily absorbed. According to Smith ('28) insoluble organic aluminum compounds are commonly formed, but even when they are absorbed the aluminum does no damage, circulates freely and is eliminated at such a rate that it does not accumulate. The presence of free phosphate in the intestine as in these diets must lead to insoluble aluminum phosphate thus making the diet equivalent to diet 16 at a lower level.

Under diet 27 parathyroidectomy removes a potentially much larger gland. Serum calcium drops to 4.24 mg./100 cc. while the phosphorus level rises very high. Under this stimulus the fetal parathyroid doubles in weight and calcium storage drops to 44% in pups 67% normal size. Addition of viosterol to this diet produces a slight hypercalcemia and protects the maternal parathyroid against enlargement; it does not protect so completely the fetal parathyroid after maternal parathyroidectomy, since this organ increases 35% in weight in the larger pups.

Parathyroidectomy on diet 12 removes a potentially subnormal gland, causes serum calcium to drop and phosphorus to rise and produces a fetal gland weighing the same as without parathyroidectomy. Fetuses are of normal size. Calcium storage is 76%, the highest recorded. On diet 19 a similar reaction occurs except that a potentially larger gland is removed and the fetal parathyroid is a little larger in much smaller pups. Calcium storage drops to 50% normal.

Diet 26, plus parathyroidectomy, causes a serious drop in serum calcium while phosphorus is a little above normal. This stimulus is augmented by the removal of the potentially largest maternal gland. This results in very large fetal parathyroids. Since the glands of the fetuses from normal mothers on this diet had the same value as for diet 7 the enlargement is 43%. The ability of the fetus to store calcium is 37% of normal, which is better than that for diet 10. Fetal size, however, is more than twice that observed with diet 10.

In considering the effects of parathyroidectomy we note that we have removed not only the factor which helped to maintain the maternal blood calcium level even at the expense of the skeleton, but in some cases the factor which had a positive inhibitory influence depressing the fetal glands. The production of a low maternal calcium level because of parathyroidectomy, if carried below a critical level, lowers fetal serum calcium and acts on the parathyroid as a stimulus. The effect is a summation and is very marked. The fetus is able to extract and retain calcium even against this gradient. Sato ('38) states that parathyroidectomy in the rabbit lowers the maternal serum calcium greatly but does not alter the fetal level.

Calcium and phosphorus equilibrium and parathyroid secretion are but part of the whole mechanism. Thyroid and parathyroid show evidence of being a physiologically balanced pair. With the extreme diets and in parathyroidectomy the thyroid is certainly involved though a proper method of measurement of the effect is not yet clear. Diet 26 continued through pregnancy and lactation produces in the young a

thyroid structure much like that of Graves' disease. Chandler and Pickett ('40) show that parathyroidectomy does not lower oxygen consumption while it lowers serum calcium, and Boelter and Greenberg ('41) found that lowering serum calcium raises the rate of basal metabolism. Canterow, Stewart and Housel ('38) showed that in dogs hyperparathyroidism was associated with thyroid alteration, and Sinclair ('41) found in a cadaver thyroid hyperplasia associated with two parathyroid tumors. Aub and associates ('29) reported that hyperthyroidism increased calcium loss through both the kidneys and intestine. Halstead (1896) found that after nearly complete thyroidectomy in dogs the pups produced hypertrophied thyroid glands. It is not clear why the fetal thyroid should enlarge. Marine ('08) found that hypertrophy could be prevented by giving the mother either iodine or thyroxine. He thought the fetal gland contained no iodine. Hudson ('31) showed that the thyroxine content of the fetal thyroid could be altered by dosing the mother with iodine but while the mother's thyroxine-iodine content varied between 11 and 127 μg. the fetal secretion did not vary outside the limits 13 to 26 μg. per 100 mg. of thyroid tissue. This illustrates the resistance of the fetus to forced changes.

While this series of experiments has yielded results, most of which are consistent, it has also raised new problems by showing the unsuspected cumulative effects of successive pregnancies on the maternal parathyroid with no similar effects on successive litters. It shows a positive correlation of fetal body and gland weights on a normal diet but this correlation does not hold for other diets especially after parathyroidectomy. In the adult there is no such correlation. It has demonstrated that the fetal gland like the rest of the fetal body is resistant to outside influences but is dependent on the maternal organism for its supplies and is capable of giving a differential response to large variations in that supply. The glands function early and effectively. Although Werelius ('13) had suggested that the fetal parathyroid did not function, Sato ('38) demonstrated that an extract from the fetal calf parathyroid is effective in the rabbit.

## CONCLUSIONS

1. Fetal rat parathyroids constitute, at birth, 2.8 mg./Kg. of body weight, which is just about double the ratio in adult rats. Diet alone can force variations from a low 2.14 to high 3.4 and maternal parathyroidectomy extends this group variation from 2.6 to 10.34 mg./Kg. The extreme parathyroid weight ratio produced in the maternal rat after six pregnancies was 12 mg./Kg.

2. Fetal parathyroids in rat pups at term are affected by diet through changes in the maternal serum calcium and phosphorus levels and through the stimulus of excessive maternal parathyroid hormone.

3. A diet balanced in calcium and phosphorus content was accepted, because of its high coefficient of reproduction, as an adequate or even optimum diet. It failed to prevent parathyroid enlargement during pregnancy with cumulative effects in a second pregnancy.

4. A diet containing more calcium and less phosphorus was found to suppress both maternal and fetal parathyroids in normal rats but gave results superior to the balanced diet after parathyroidectomy. If extremely unbalanced, fetal calcium storage suffers badly. Aluminum acetate added to a normal diet acted exactly like a hypercalcemic diet.

5. The fetal parathyroids are depressed by an excess of maternal serum calcium or an excess of parathyroid hormone from an enlarged maternal gland.

6. The fetal parathyroids are stimulated by subnormal maternal serum calcium or an excess of serum phosphorus. Both of these conditions are exaggerated in parathyroidectomy.

7. The fetal parathyroid is, at its largest, only about 5% of the adult size and does not protect the mother against the results of parathyroidectomy. The fetal hormone is not lost by dilution but is able to protect fetal serum calcium levels.

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# THE NEED FOR PANTOTHENIC ACID AND AN UNIDENTIFIED FACTOR IN REPRODUCTION IN THE DOMESTIC FOWL

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The importance of pantothenic acid in the nutrition of the chick is well established. Ringrose, Norris and Heuser ('31) suggested that vitamin G was a complex consisting of at least two components, one of which was essentially a growth-promoting factor and the other necessary for the prevention of chick dermatosis. The latter component was subsequently termed the chick antidermatosis vitamin or "filtrate factor." The identity of the antidermatosis vitamin and pantothenic acid was established simultaneously by Jukes ('39 a) and Woolley, Waisman and Elvehjem ('39).

Phillips and Engel ('39) studied the histopathology of pantothenic acid deficiency in the chick and found this vitamin to be necessary for the prevention of thymus involution, liver damage, keratitis and dermatitis of the skin and neuropathology of the spinal cord. Snell, Pennington and Williams ('40) showed that the pantothenic acid content of chick tissues is much greater when the chicks are fed an adequate diet than when they are fed a diet deficient in this vitamin. Studies on the quantitative requirement of chicks for pantothenic acid have been made by Jukes ('39 b) and by Bauernfeind, Norris and Heuser ('41).

The role of pantothenic acid in the nutrition of the hen is not as clearly established as it is in the nutrition of the

chick. Lepkovsky, Taylor, Jukes and Almquist ('38) concluded that the antidermatosis vitamin exercised no function in maintaining either hatchability, egg production or character of embryonic mortality under the conditions of their experiments. Bauernfeind and Norris ('39), on the other hand, reported that a heat-labile, non-adsorbable factor in rice bran filtrate, believed to be identical with the antidermatosis vitamin, is required for reproduction in hens fed a so-called heated diet supplemented with heated liver extract. Failure in reproduction occurred, however, unless this diet was supplemented with a heat-labile, adsorbable factor subsequently designated "factor R." The original factor R has since been shown by Schumacher, Heuser and Norris ('40) to consist of at least two factors, termed factors R and S.

The results of Lepkovsky and associates ('38) showed, however, that the amount of the antidermatosis vitamin in eggs is dependent on the amount in the diet of the hens, and is, therefore, important in the production of chicks resistant to dermatosis. Bauernfeind and Norris ('39) also showed that the amount of the vitamin in the egg, as determined by the resistance of newly hatched chicks to the development of dermatosis, is dependent on its concentration in the diet of the hen. Employing synthetic calcium pantothenate, Snell, Aline, Couch and Pearson ('41) demonstrated that the pantothenic acid content of eggs is directly proportional to the amount present in the diet.

More exact studies on the role of pantothenic acid in nutrition were made possible by both the establishment of its chemical structure and its synthesis by Williams and Major ('40), since this brought about the commercial production of this vitamin. It was decided, therefore, to determine whether or not the heat-labile, non-adsorbable factor reported by Bauernfeind and Norris ('39) is identical with pantothenic acid, and also to study further its role in reproduction in hens. The results of this investigation are presented in this report.

## EXPERIMENTAL PROCEDURE

Single Comb White Leghorn pullets reared on an adequate diet were used as the experimental animals in this study. They had been laying for several weeks prior to the beginning of the investigation. Five lots of twelve pullets each were selected for uniformity in egg production, body weight and general physical condition. They were placed in pens equipped with wire floors in order to prevent coprophagy and received identical management, other than dietary, throughout the experiment. One male was placed in each pen at the start of the experiment. Subsequently all males were rotated frequently among the different pens in order to eliminate differences in breeding capacity. The hens were weighed at 2-week intervals, and the eggs were incubated weekly. During incubation the eggs were candled on the seventh, fourteenth and eighteenth days in order to determine fertility and embryonic mortality. In cases of doubtful fertility the eggs were broken and examined.

The composition of the basal diet used in this experimental work is given in table 1. The ration was a modification of the

TABLE 1

*Basal diet.*

| INGREDIENT | gm. |
|---|---|
| Heated yellow corn meal | 42.5 |
| Heated wheat flour middlings | 20.0 |
| Heated wheat bran | 20.0 |
| Purified casein | 8.0 |
| Soybean oil | 2.5 |
| Cod liver oil (1500 I.U. vitamin A, 125 I.U. vitamin D) | 0.5 |
| Wheat germ oil | 0.5 |
| Pulverized limestone | 3.5 |
| Steamed bone meal | 2.0 |
| Salt | 0.5 |
| Riboflavin | 0.0275 |
| Thiamine | 0.0150 |
| Nicotinic acid | 0.0300 |
| plus | |
| Concentrate of factors R and S equivalent to 5.0% of yeast | |

one reported by Bauernfeind and Norris ('39) and contains an adequate amount of all the known vitamins, other than pantothenic acid, needed to support good hatchability. The cereal portion of the diet was heated in an oven for 36 hours at a temperature of 120°C. prior to being mixed with the other ingredients. This treatment has been shown by Kline, Keenan, Elvehjem and Hart ('32) to destroy most of the pantothenic acid. The casein used in the diet was purified in order to free it of pantothenic acid and other water-soluble vitamins. All diets were mixed at intervals of not longer than 2 weeks in order to prevent destruction of any essential nutritive factor by storage.

When the basal diet was fed to chicks, lesions of the dermatosis typical of pantothenic acid deficiency developed. The syndrome was cured by the addition of pantothenic acid to the diet. A microbiological assay of the diet by the procedure of Pennington, Snell and Williams ('40) showed that it contained approximately 225 μg. of pantothenic acid per 100 gm., which is in agreement with previous observations by this laboratory. This is greatly below the requirement of the chick according to Jukes ('39 b) and Bauernfeind, Norris and Heuser ('41).

The basal diet used originally by Bauernfeind and Norris ('39) contained as a source of riboflavin a liver extract which was heated with the cereals. Later work conducted at this laboratory indicated that this extract was essential for reproduction in hens fed the heated diet. Therefore, it was decided to investigate further the possibility that the liver extract used in the diet contained some unrecognized factor which plays an essential role in reproduction. All liver extract [1] used in the experiment was mixed with a small amount of the unheated cereals and heated in an oven for the same length of time as the cereals. After the heat treatment it was finely ground and mixed at the desired level with the other ingredients. By heating in this way there was eliminated the

[1] Wilson's 95% alcohol soluble liver extract paste.

possibility that any markedly beneficial effect from the liver extract was due to additional pantothenic acid.

Factors R and S used in the basal diet were prepared from yeast according to the method described by Schumacher, Heuser and Norris ('40). Factor S was precipitated in alcohol solution at pH 1 and factor R at pH 7. The filtrate was freed of alcohol and concentrated to a suitable volume. The concentrate was used in these studies, since it had not been determined previously whether or not it contained any additional factor, or factors, benefiting reproduction. It is referred to hereafter as the "yeast filtrate." The yeast filtrate was mixed with the cereals and heated in the same manner as the liver extract.

All lots were initially placed on the basal diet for a preliminary period of 8 weeks (period 1) in order to deplete them of their reserve of pantothenic acid. This depletion period was followed by two experimental periods of 10 weeks each (periods 2 and 3) during which time the different lots received pantothenic acid, liver extract and yeast filtrate in various combinations and amounts. The pantothenic acid used in this experimental work was supplied in the form of synthetic calcium pantothenate, dextrorotatory.[2]

## RESULTS AND DISCUSSION

The results of the experimental work on reproduction are summarized in table 2. At the end of 4 weeks' depletion, during which all lots of birds received the basal diet, the hatchability was nearly zero in all cases. After 8 weeks' depletion the hatchability of all groups of eggs was zero. The basal diet was markedly deficient, therefore, in a factor, or factors, necessary for reproduction.

During period 2 the hatchability of the eggs from lot 1, receiving the basal ration, as well as those from lot 4, receiving the yeast filtrate and heated liver extract, was zero. This showed that these supplements alone were ineffective in promoting hatchability when added to the basal diet.

[2] Merck.

On the other hand, the hatchability of the eggs from lot 2, receiving pantothenic acid in addition to the heated liver extract, gradually increased until it reached a level of 50–60% which was maintained with some variation until the end of the period. The eggs from lot 5, which received yeast filtrate in

TABLE 2

*Per cent hatchability of fertile eggs.*[1]

| WEEK | LOT 1 | LOT 2 | LOT 3 | LOT 4 | LOT 5 |
|---|---|---|---|---|---|
| | | Period 2 [2] | | | |
| | Basal | Pantothenic acid + liver extract | Pantothenic acid + yeast filtrate | Liver extract + yeast filtrate | Pantothenic acid + liver extract + yeast filtrate |
| 9 | 0.0 | 5.0 | 0.0 | 0.0 | 5.0 |
| 10 | 0.0 | 31.0 | 0.0 | 0.0 | 0.0 |
| 11 | 0.0 | 34.8 | 7.2 | 0.0 | 53.6 |
| 12 | 0.0 | 48.0 | 27.7 | 0.0 | 56.0 |
| 13 | 0.0 | 55.0 | 18.2 | 0.0 | 53.0 |
| 14 | 0.0 | 58.0 | 26.0 | 0.0 | 50.0 |
| 15 | 0.0 | 53.0 | 27.0 | 0.0 | 42.0 |
| 16 | 0.0 | 59.1 | 20.0 | 0.0 | 47.6 |
| 17 | 0.0 | 50.0 | 42.9 | 0.0 | 52.2 |
| 18 | 0.0 | 46.3 | 34.2 | 0.0 | 58.1 |
| 12–18 inc. | 0.0 | 52.8 | 28.0 | 0.0 | 51.3 |
| | | Period 3 [3] | | | |
| | Pantothenic acid | Liver extract | Basal | Pantothenic acid + liver extract | Pantothenic acid |
| 19 | 2.3 | 71.0 | 24.4 | 19.2 | 68.8 |
| 20 | 13.3 | 55.9 | 18.2 | 79.1 | 61.3 |
| 21 | 12.5 | 50.0 | 16.3 | 63.2 | 60.8 |
| 22 | 12.6 | 47.2 | 20.0 | 57.2 | 21.9 |
| 23 | 14.3 | 25.7 | 0.0 | 59.3 | 17.4 |
| 24 | 16.6 | 19.5 | 0.0 | 50.0 | 38.7 |
| 25 | 12.5 | 17.1 | 0.0 | 67.9 | 36.8 |
| 26 | 14.2 | 17.3 | 0.0 | 60.7 | 34.2 |
| 27 | 9.7 | 0.0 | 0.0 | 54.5 | 35.1 |
| 28 | 13.8 | 0.0 | 0.0 | 65.2 | 30.5 |

[1] During the depletion period (period 1) all lots were fed the basal ration. At the end of the eighth week the hatchability of fertile eggs for all lots was zero.

[2] During period 2 the dietary additions per 100 gm. of diet were as follows: 1.0 mg. calcium pantothenate, dextrorotatory; 0.5 gm. liver extract; yeast filtrate equivalent to 5 gm. of yeast.

[3] During period 3 the calcium pantothenate was increased to 2.0 mg. and the liver extract to 2.0 gm. per 100 gm. of diet.

addition to pantothenic acid and liver extract responded in a fashion similar to those from lot 2. It was evident, therefore, that the heated yeast filtrate contained no additional factor influencing hatchability. The hatchability of the eggs from lot 3, which received the pantothenic acid and yeast filtrate, was approximately 25% compared to zero for those from lot 1, but it did not equal that of the eggs from the lots which received pantothenic acid and liver extract. The hatchability of the eggs from lot 5, which received pantothenic acid, liver extract and yeast filtrate, was approximately the same as that of the eggs from lot 2, receiving pantothenic acid and liver extract but no yeast filtrate.

It is evident from these results that pantothenic acid is necessary for reproduction in hens. It is also apparent that except when heated liver extract was included in the diet, pantothenic acid was unable to raise the hatchability above an average of about 25%. Therefore, the liver extract used in this experimental work contained an additional factor, or factors, required for reproduction. No evidence was obtained that the yeast filtrate influenced hatchability favorably.

The experimental setup for period 3 was designed to confirm and supplement the conclusions drawn from the previous period. The level of synthetic calcium pantothenate fed was increased from 1.0 mg. per 100 gm. of diet to 2.0 mg., and the liver extract was increased from 0.5 gm. to 2.0 gm. The use of the yeast filtrate was discontinued in this period, since it was found previously to be ineffective in promoting hatchability when added to the basal diet along with pantothenic acid and liver extract.

The addition of pantothenic acid to the diet of lot 1, which had received the basal diet only for the preceding 18 weeks and which had produced no chicks for the preceding 14 weeks, immediately increased hatchability to 12–15% where it remained for the rest of the period. This increase in hatchability can be attributed only to the addition of pantothenic acid. The hatchability of the eggs from lot 4, now being fed pantothenic acid in addition to the liver extract that it was

already receiving, increased from zero to approximately 80% in 2 weeks. The hatchability of the eggs from this lot subsequently declined slightly but remained in general above that of the eggs from lots 2 and 5 in period 2. It is possible that this was due to the increased levels of the heated liver extract and pantothenic acid supplied in period 3.

When pantothenic acid was removed from the diets of the birds in lots 2 and 3, the hatchability of their eggs dropped to zero. The decline, however, was much slower in the case of the eggs from the lot receiving the 2.0% of liver extract. This may have been due either to the presence of small amounts of undestroyed pantothenic acid in the heated liver extract or to a sparing effect of the factor, or factors, in liver extract upon pantothenic acid.

The hatchability of the eggs of lot 5, which had received liver extract and pantothenic acid in the previous period and had maintained a hatchability of about 50%, declined to approximately 30% in period 3, after removal of liver extract from the diet. Although the hens of both lots 1 and 5 received only pantothenic acid during the third period, the hatchability of the eggs of lot 5 was significantly better than that of lot 2. This is believed to be due to the fact that during the previous period of 10 weeks the birds of lot 5 received liver extract while those of lot 1 had been fed for 18 weeks on the basal diet only.

The results of the third period confirmed those of the second period showing that pantothenic acid is essential for hatchability and that heated liver extract contains another factor, or factors, required for this purpose. The results indicate, moreover, that the basal diet was incomplete even when supplemented with both pantothenic acid and liver extract, as the hatchability of the eggs from a lot of hens maintained under the same conditions as the experimental hens but receiving a normal diet was approximately 72%. This was above the average hatchability for the lots receiving pantothenic acid and liver extract.

The rate of egg production in all lots decreased from approximately 60% to about 40% at the end of the 8 weeks' depletion period. The rate of egg production of lot 1, which received the basal diet for an additional 10 weeks, made only a slight additional decrease during this time. The rate of production of all lots increased when the supplements were added to their diets. The increases, however, were variable and not always consistent. They were most noticeable when both the pantothenic acid and heated liver extract were added to the diets. It appears from these results, therefore, that these factors favorably influence egg production but that the effect is not great under the conditions of the experiment.

After the hens had been fed the depletion diet for about 7 weeks, it was noticed that about 80% of the birds developed a mild form of dermatosis on the feet and lower shanks. The syndrome was similar to that encountered at times in chicks fed a diet deficient in pantothenic acid but in most cases it was not as severe. It was characterized by cracks and fissures on the bottoms of the feet and by peeling of the scales on the lower shanks and tops of the toes which was profuse at times. No dermatosis developed either at the angles of the mandibles or on the margins of the eyelids. A group of pullets kept under identical conditions but receiving a normal diet did not exhibit these lesions. Gradual recovery from the dermatosis was noted when pantothenic acid was added to the diet. Complete recovery resulted in many cases by the end of the experiment. At this time several of the hens which had not yet recovered were placed on a normal diet and given massive oral doses of pantothenic acid. Recovery was more rapid under these conditions.

All of the hens fed the basal diet lost weight appreciably and failed to regain their normal weight even when the diet was supplemented with both pantothenic acid and heated liver extract. This is further evidence that there is still another deficiency in the basal diet used in this experimental work.

The mortality of the hens of lots 2, 3 and 5 was 16.6% in each case and that of lots 1 and 4 was 33.3%. The latter two

lots received no supplementary pantothenic acid for the first 18 weeks of the experiment. The greater portion of the mortality occurred during this time. The lack of this vitamin, therefore, may have been a contributing cause in the higher mortality of these two lots.

## SUMMARY

Evidence is presented which shows that pantothenic acid is required for reproduction in hens. It was found that for this function they require also an unidentified, heat stable factor, or factors, present in liver extract. The addition of pantothenic acid alone to the basal diet increased hatchability from zero to 10–30%. When it was added to the basal diet along with the factor, or factors, in liver extract, hatchability was increased to 50–60%. The factor, or factors, in liver extract had little or no influence on hatchability except in the presence of pantothenic acid. A dermatosis was observed on the feet and shanks of hens fed a diet deficient in pantothenic acid.

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# THE INACTIVATION OF VITAMIN $B_1$ IN DIETS CONTAINING WHOLE FISH

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TWO FIGURES

(Received for publication August 22, 1941)

A nutritional disease of foxes induced by feeding diets that contain uncooked whole fish has been described under the name of "Chastek paralysis" (Green, '36). In 1937 we described the microscopic pathology of this disease, pointed out the relationship to the feeding of whole uncooked fish, and expressed our opinion from evidence at hand that the disease was caused by a vitamin-$B_1$ deficiency (Green, '37; Green et al., '37).

In 1938 Carlström and Jonsson reported an outbreak of an acute disease of foxes in Sweden characterized by loss of appetite, ataxia, and acetonemia. Twelve foxes died in a period of 2 weeks on a ranch where a diet of fish and cooked cereals was used. A ration containing yeast stopped the outbreak. These authors believed the disease to result from a vitamin-$B_1$ deficiency but did not recognize fish as a causal factor. Apparently they were dealing with Chastek paralysis.

Ender and Helgebostad ('39) also appear to have recognized Chastek paralysis in foxes, minks, and ferrets on fur farms

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in Norway. They believed the disease to be due to a vitamin-$B_1$ deficiency and termed the condition beriberi but did not recognize fish as a factor of importance. By feeding silver foxes a diet of casein, cornstarch, vegetable fat, salt mixture, autoclaved yeast, dried whale meat, cod liver oil, vitamin C, and calcium carbonate, they succeeded in producing an illness similar to that seen on fur farms. Lunde ('39) opposed the ideas of Green that fish was a causative factor and that the condition was fundamentally a vitamin-$B_1$ deficiency. More recently Kringstad and Lunde ('40) have stated that silver foxes given a ration deficient in vitamin $B_1$ developed anorexia with loss of weight, followed by convulsions 3 or 4 weeks later. Synthetic vitamin $B_1$ in the amount of 70 international units a day prevented the symptoms. Injections of thiamine were curative.

In 1940 we (Green and Evans) published a summary of our early findings including a list of the species of fish found to cause the disease, and a brief description of the characteristic pathologic changes in the brain of affected foxes. Coombes ('40) has observed Chastek paralysis and accepted our finding that it is related to the feeding of fish. He considers it as a poisoning and not a deficiency disease. In a recent publication we (Green, Carlson and Evans, '41) demonstrated that Chastek paralysis can be prevented by feeding adequate amounts of thiamine hydrochloride in a fish diet, and confirmed our earlier finding that the disease is a $B_1$ avitaminosis induced by including whole fish of certain species in a fully adequate ration. We have further shown (Evans, Carlson and Green, in press) that characteristic lesions are present in the central nervous system. The lesions have the distribution and the histologic features which in man are considered typical of Wernicke's hemorrhagic polioencephalitis. In the present paper are reported experiments (1) that demonstrate quantitatively the destruction of thiamine by whole fish and (2) that yield information on the anatomical distribution in the fish of the thiamine-inactivating factor.

*Quantitative tests of the amount of thiamine inactivated by a known amount of fish*

In order to determine approximately the amount of thiamine required to prevent the occurrence of Chastek paralysis in foxes given a ration containing a known amount of fish, we have performed an experiment utilizing fifty red fox pups about 6 months old. The percentage composition of the basal diet in this experiment was the same for all animals; namely, cereal 15, bread 10, carrots 9.5, horse meat 45, whole carp 20, cod liver oil 0.5. The cereal was a mixture of wheat, corn, oats and rice. All ingredients were ground together to make a mixture the consistency of hamburger. Each fox received 12 ounces of feed a day. Thiamine hydrochloride in specified amounts was added as an aqueous solution and thoroughly mixed with all other ingredients of the ration.

The foxes were divided into five groups of ten animals each. Four of the groups were fed daily rations containing 10, 5, 2, and 1 mg. of thiamine hydrochloride, respectively. Foxes in the fifth group received the basal ration without a supplement of thiamine. The experiment was begun on September 4, 1940, and was terminated on December 5. If foxes with severe symptoms did not die quickly, they were etherized. As soon as half the animals in any group had died of Chastek paralysis or had been killed in extremis, the surviving foxes in that group were taken off the experiment. The results obtained in the experiment are shown in figure 1.

We had previously found that 25 mg. of thiamine a day would protect foxes from Chastek paralysis while on a diet containing 20% whole uncooked carp. In the present experiment 10 mg. of thiamine likewise afforded complete protection against the disease. The inclusion of 5 mg. of thiamine in the diet offered complete protection from death and from any neurologic symptoms whatsoever. However, after 7 weeks some animals went off feed at irregular intervals. Loss of appetite is characteristically the first evidence of Chastek paralysis, and it is our opinion that the inappetence presented

by the foxes in group II, fed 5 mg. of thiamine, is a manifestation of subclinical Chastek paralysis. Among the foxes that received 2 mg. of thiamine, inappetence and subsequent fatalities appeared a month later than in the controls. The experiment was terminated for group III after five deaths occurred, and there is no doubt that all animals would have succumbed had the experimental diet been continued. Some slight beneficial effect was produced in group IV by adding 1 mg. of thiamine,

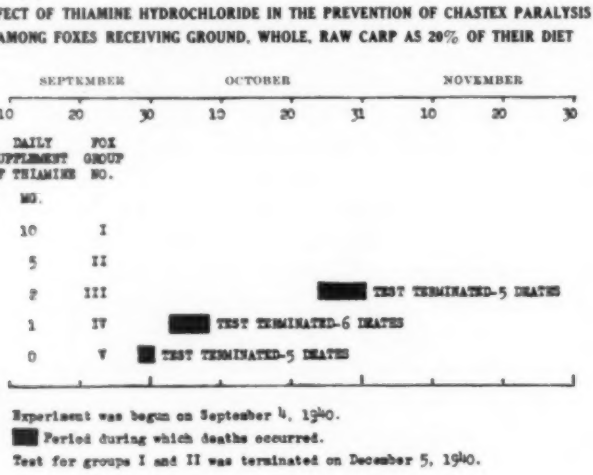



Figure 1

inasmuch as Chastek paralysis appeared a week later in that group than in the control foxes, which received no supplement of thiamine. Although symptoms and fatalities were delayed in the groups given 1 mg. and 2 mg. of thiamine, the disease, when it did appear, was as acute and as typical pathologically as it was in the controls.

As each fox received approximately 2.4 ounces of whole carp daily in the one meal offered, we can determine roughly the amount of thiamine inactivated by a pound of carp and the interrelationship between the degree of inactivation and the

disease produced at the different levels of thiamine fed (table 1). We have no accurate knowledge of the thiamine content of the basal diet; but if frozen horse meat has approximately the same thiamine content as lean beef, there were probably about 50 I.U. of thiamine in each 12-ounce feeding. Our estimates of the amount of thiamine inactivated by a pound of carp are conservative, since they take into account only the thiamine added as a supplement to the ration.

TABLE 1

*The inactivating effect of one pound of carp on different amounts of thiamine.*

| FOX GROUP NO. | DAILY THIAMINE SUPPLEMENT | AMOUNT OF THIAMINE TO EACH POUND OF CARP [1] | | EFFECT ON FOXES |
|---|---|---|---|---|
| | | Weight | International units | |
| | *mg.* | *mg.* | | |
| I | 10 | 67.0 | 22,000 | No deaths; no symptoms in 92 days. |
| II | 5 | 33.5 | 11,000 | No deaths; mild symptoms a month after controls died. |
| III | 2 | 13.4 | 4,400 | Deaths delayed 1 month. |
| IV | 1 | 6.7 | 2,200 | Deaths delayed 1 week. |

[1] Figures indicate the amount of thiamine added to 5 pounds of ration (each 5 pounds of ration contained 1 pound of carp). The relatively small amount of thiamine occurring naturally in the basal diet (see text) is not included in these figures.

From the data shown in table 1 it is obvious that when fish is fed as 20% of the ration, 1 pound of carp will inactivate supplemental thiamine in excess of 4,400 units; but 1 pound of carp will not completely inactivate 22,000 units.

The actual amount of thiamine that a pound of carp inactivates is not a constant value but is variable, depending upon the amount of thiamine mixed with the fish. One pound of carp apparently failed to inactivate completely 2,200 units of thiamine in the case of group IV, yet a like amount of carp in group II must have inactivated about 10,000 units. This dependence of the amount of thiamine inactivated upon the

amount of thiamine available suggests adsorption as the basis of the inactivation, rather than a simple chemical reaction between two substances. It should be emphasized that the term inactivation is used only to describe the gross result of feeding carp and thiamine in the same ration. The actual physical, chemical, and physiologic phenomena concerned are still a matter for conjecture.

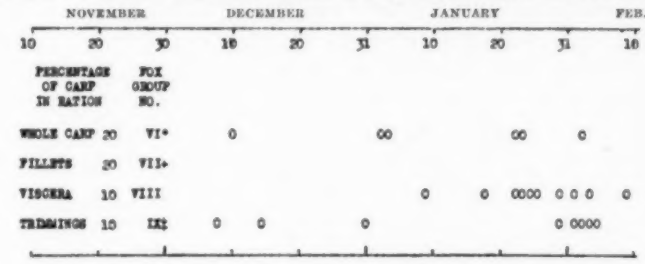
### *Study of the potency of various organs of fish in producing Chastek paralysis*

In order to determine which organs of fish contain the substance that causes Chastek paralysis, an experiment in which forty red foxes were used, was begun in November, 1940. The carp fed in this experiment were freshly seined. Some were used as whole fish; others were eviscerated and skinned, after which the muscle was removed from the skeleton as fillets. The gastro-intestinal tract, liver and other viscera were frozen in one container; the fillets, in a second; and the heads, fins, tails, and skin, with one-fourth of the original amount of scales, were frozen in a third container. The basal diet in this experiment had the following percentage composition: cereal 15, bread 10, carrots 9.5, cod liver oil 0.5, horse meat and fish 65. Through an error, fresh unfrozen horse meat was used instead of frozen horse meat during the first month of this experiment. On December 13 fresh meat was discontinued and the frozen product was used thereafter. In figure 2 are listed the fish ingredients of the diet given each group of ten foxes and the results obtained. All foxes that died displayed typical symptoms of Chastek paralysis. The experiment was begun on November 13, 1940, and terminated on February 26, 1941, when all the foxes fed viscera and all those fed trimmings (heads, skin, fins and skeleton) had succumbed to the disease.

It is clear from the results that the agent in whole carp which is responsible for Chastek paralysis is found in the external structures of the fish and in the viscera, but is not present to any comparable degree in the somatic muscle. It was also apparent during the course of the experiment that

the carp viscera and trimmings were fully as effective as whole fish, or more effective than whole fish, in the production of Chastek paralysis. At the time the experiment was terminated, only six of the ten foxes that received whole fish had succumbed, while the foxes that received viscera and trimmings had all died. Three cases of Chastek paralysis developed while the foxes were receiving fresh, unfrozen horse meat. The greatest grouping of deaths came around January 25, 2½

**POTENCY OF DIFFERENT ANATOMIC FRACTIONS OF CARP IN PRODUCING CHASTEX PARALYSIS**




Experiment was begun on November 13, 1940, and terminated on February 26, 1941.
Fresh, unfrozen horse meat was used until December 13; frozen horse meat was used after that date.
0 - Death of a fox.
* Six of the 10 animals in this group died of Chastek paralysis or were killed in extremis. The remaining 4 foxes were injected with thiamine on February 26 and placed on a diet that contained liver but no fish.
+ Foxes receiving the carp fillets remained entirely well.
‡ Two remaining foxes in this group died on February 26, 1941.

Figure 2

months after fish was added to the ration and 6 weeks after the substitution of frozen horse meat for the fresh product. The quick and decisive development of Chastek paralysis by the foxes that received whole fish in the first experiment (fig. 1) was not at all duplicated by the foxes in the second experiment (fig. 2) that were fed whole fish. The experimental details were identical for the two groups except for the period in which fresh horse meat was fed to the latter group of foxes.

## DISCUSSION

From our series of experiments on Chastek paralysis, it seems established that the disease is not analogous to beri-

beri, which is known to result from a deficiency of several vitamins, but rather to Wernicke's disease in which there is probably a deficiency of only vitamin $B_1$ (Alexander, '40). The regularity with which we have been able to prevent the development of Chastek paralysis by the use of crystalline thiamine indicates that in Chastek paralysis the deficiency concerns only this one substance, and that other vitamins are present in an adequate amount.

Recently, György, Rose, Eakin, Snell and Williams ('41) have presented experimental evidence that "egg-white injury" results from the inactivation of biotin by a component of egg white which they call "avidalbumen". The mechanism they describe closely parallels that which we postulated as operative in Chastek paralysis. The similarity extends even to the fact that the noxious substance in egg white, like that in fish, is destroyed by cooking. Complete proof has not yet been obtained, but the most probable basis for each disease appears to be the inactivation of a specific vitamin by a constituent of the diet.

Beck and Peacock ('41) have carried out dietary investigations in relation to papillomatosis of the fore-stomach of the rat and have concluded that "the mechanism of papillomatosis in these experiments seems to be related to an induced avitaminosis-A due to the presence in repeatedly heated fats of an 'anti-vitamin A' of undetermined nature".

Mackenzie, Mackenzie and McCollum ('41) have shown that the muscular dystrophy induced by feeding cod liver oil to herbivores (rabbits, in their experiments) can be prevented by giving the animals very large doses of alpha-tocopherol.

Apparently the adequacy of a given amount of vitamins A and E, as well as of biotin and thiamine, can be unfavorably affected by other ingredients of the diet. The possibility that a dietary constituent may destroy a given vitamin or in some other way cause a normally adequate supply of that vitamin to become insufficient may be a factor of greater importance in human and veterinary medicine than has been generally recognized.

Certain fur farmers have fed whole carp without untoward results by giving the animals chunks of carp 1 day, and giving them a balanced ration without whole fish the following day — alternating the fish and other feed in a systematic manner. In fact, some fur farmers have used whole carp with evident success by giving the fish alone in chunks in the morning and giving a balanced ration as a second or evening meal. On ranches on which fish has been mixed with the total diet but fed only 3 or 4 days a week, no difficulty with Chastek paralysis seems to have been encountered. Although it takes very large amounts of thiamine mixed with fish to prevent Chastek paralysis, a relatively small amount of vitamin $B_1$ consumed at a time when it will not be mixed with the fish either in the feed pan or in the gastro-intestinal tract is evidently sufficient to prevent the development of Chastek paralysis. This point is of considerable practical importance. It has not yet been experimentally tested but seems fairly well established on the basis of ranch observations.

## SUMMARY

1. Among foxes fed a ration containing 20% whole carp and variable amounts of thiamine, Chastek paralysis failed to develop in those given 10 mg. of thiamine each day, and manifested itself only as transient anorexia among those given 5 mg. of thiamine. The onset of Chastek paralysis was delayed 1 month among foxes given 2 mg. of thiamine hydrochloride each day, and then appeared in this group in the usual fatal form.

2. A group of foxes fed skin, scales, fins, skeletons and heads of carp developed Chastek paralysis; another group fed the carp viscera contracted the disease; those given muscle as carp fillets showed no evidence of Chastek paralysis.

3. Observation on fur ranches where fish has been used indicates that Chastek paralysis will not develop if whole fish is fed several days a week and a diet containing adequate amounts of vitamin $B_1$ is fed on the other days of the week.

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# BIOTIN IN CHICK NUTRITION[1]

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In a previous paper (Hegsted, Oleson, Mills, Elvehjem and Hart, '40) we described a typical scaly dermatitis in chicks fed purified rations, which condition was not prevented by pantothenic acid. Distribution and properties of the protective factor agreed with those reported for vitamin H, and potent preparations of vitamin H produced cures of the dermatitis. György, Melville, Burk and du Vigneaud ('40) first noted the possible identity of biotin and vitamin H. A short while later du Vigneaud, Melville, György and Rose ('40) demonstrated the vitamin H activity of crystalline biotin methyl ester in rats showing egg white injury. Purification of du Vigneaud's crystalline biotin methyl ester to constant melting point and constant biotin activity produced crystals of such potent vitamin H activity that there was no longer any doubt that biotin and vitamin H were the same compound (György, Rose, Hofmann, Melville and du Vigneaud, '40). Various biotin preparations have subsequently been used in studies of the dermatitis. Ansbacher and Landy ('41) found that crystalline biotin methyl ester completely cured a severe dermatosis similar to that described by us, but produced on a heated ration.

We wish to present here further studies on the dermatitis as produced on purified rations. Both biotin concentrates and

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the Wisconsin Alumni Research Foundation and the Works Progress Administration.

free crystalline biotin have prevented the condition and promoted growth in chicks.

## EXPERIMENTAL

The basal rations used have had the following percentage composition: dextrin 55, purified casein 18, salts IV (Hegsted, Mills, Elvehjem and Hart, '41) 5, defatted cartilage 15, soybean oil 5, and solubilized liver extract 2. Each kilo of ration contained inositol 1 gm., thiamine 3 mg., pyridoxine and riboflavin 4 mg. each, calcium pantothenate 15 mg., nicotinic acid 100 mg. and choline chloride 1.5 gm.[2] In certain of the groups receiving molasses the solubilized liver extract was omitted and in others a concentrate prepared from solubilized liver extract (Hutchings, Bohonos, Hegsted, Elvehjem and Peterson, '41) was substituted for it. Each chick received 2 drops of fortified haliver oil per week, supplying adequate amounts of vitamins A and D.

Day-old White Leghorn chicks were used. They were kept on raised screens in small heated brooders and given the experimental ration and water ad libitum.

## RESULTS

As previously reported, lesions started to appear at 2–3 weeks and were quite severe at 4 weeks. The syndrome is characterized by lesions first appearing on the bottoms of the feet, followed by mandibular lesions. About 80% of all the chicks on the basal ration showed the dermatitis at 4 weeks.

Table 1 shows the effectiveness of various supplements in preventing the dermatitis. Molasses, a fair source of biotin, prevented it almost completely at a level of 10% of the ration. Biotin concentrates[3] also were effective. A level of 5 μg. of

[2] We are indebted to Merck and Co., Inc., Rahway, N. J., for the thiamine, pyridoxine, riboflavin, calcium pantothenate, nicotinic acid and choline chloride; to the Wilson Laboratories, Chicago, Ill., for the solubilized liver extract and cartilage; to Allied Mills, Peoria, Ill., for the soybean oil; and to Abbott Laboratories, North Chicago, Ill., for the haliver oil.

[3] Generously supplied by Dr. Maurice Landy of the Research Laboratories, S.M.A. Corporation, Chagrin Falls, Ohio.

biotin in the form of this concentrate per 100 gm. of the ration was only partially effective — of the six chicks, three had slight lesions. Levels of 10 and 20 μg. per 100 gm. completely prevented the dermatitis. One group of chicks was given free crystalline biotin (du Vigneaud)[4] at a level of 10 μg. per 100 gm. of ration. In this group one chick showed slight lesions at 3 weeks, which were cured within a week by two injections of 2.5 μg. of the crystalline biotin. The other chicks were completely protected.

TABLE 1

*Effect of supplements on the dermatitis in chicks.*

| SUPPLEMENT TO BASAL RATION | NUMBER OF CHICKS | PER CENT WITH DERMATITIS |
|---|---|---|
| None | 95 | 80 |
| 10% molasses | 54 | 4 |
| 5 μg. biotin (S.M.A.) per 100 gm. | 6 | 50 |
| 10 μg. biotin (S.M.A.) per 100 gm. | 6 | 0 |
| 10 μg. biotin (crystalline) per 100 gm. | 3 | 0 |
| 20 μg. biotin (S.M.A.) per 100 gm. | 17 | 0 |
| 30 mg. para-aminobenzoic acid per 100 gm. | 5 | 100 |

In addition to preventing the dermatitis, biotin is also a growth factor for chicks. Those on the basal ration weighed an average of 140 gm. at 4 weeks, while those receiving 20 μg. of biotin per 100 gm. weighed 193 gm. Chicks getting 10 μg. of crystalline biotin weighed 182 gm.

Para-aminobenzoic acid neither prevented the dermatitis nor promoted growth on this ration.

Data on the biotin content of the livers and kidneys of some of the chicks showing dermatitis, and some receiving various levels of biotin are presented in table 2. Stores of biotin in the liver and kidney are very low in the chicks having dermatitis, while the biotin content of the organs of the positive controls is almost four times as great per gram. On the basis of total biotin per organ the differences are still greater because of the difference in the size of the chicks.

[4] We wish to express our appreciation to Doctor du Vigneaud, Cornell Medical School, New York, for the sample of free crystalline biotin.

TABLE 2

*Biotin content of chick tissues.*[1]

| SUPPLEMENT TO BASAL RATION | NUMBER OF CHICKS | NUMBER WITH DERMATITIS | BIOTIN CONTENT OF TISSUES-DRY | |
|---|---|---|---|---|
| | | | Liver | Kidney |
| | | | *μg./gm.* | *μg./gm.* |
| None | 10 | 10 | 3.3 | 1.3 |
| 10 μg. biotin (crystalline) per 100 gm. | 3 | 0 | 8.2 | 2.5 |
| 20 μg. biotin (S.M.A.) per 100 gm. | 5 | 0 | 12.0 | 6.0 |
| 3% kidney residue | 6 | 0 | 11.9 | 20.0 |

[1] Analyses were by the methods of Snell, Eakin and Williams ('40) and Lampen, Kline and Peterson ('41). We wish to thank Miss Margaret McGregor and Mr. J. O. Lampen for making these analyses.

## DISCUSSION

The prevention and cure of the typical dermatitis by crystalline biotin and biotin concentrates confirm our earlier observations that the dermatitis is the result of a biotin deficiency. The growth stimulating properties of biotin place it in the already long list of essential growth factors for the chick.

The biotin requirement of the chick can be roughly estimated from our data. In addition to that present in the basal ration, the minimum amount required to prevent dermatitis and give maximum growth seems to be about 10 μg. per 100 gm. of ration. Five microgram per 100 gm. is inadequate, as can be seen from the 50% incidence of the dermatitis. The one slight case at 10 μg. may indicate that this level is marginal. Molasses contains 0.7 μg. per gram so that 10% of molasses supplies 7 μg. per 100 gm., slightly below the level given above as marginal. Three per cent of kidney residue [5] in the ration has been found adequate to prevent dermatitis in thousands of chicks in other studies here. The biotin content of the kidney residue is about 2.5 μg. per gram, so that 7.5 μg.

[5] The residue after exhaustive extraction of defatted kidney with water, 50% ethanol, and 50% methanol.

per 100 gm. is supplied by it. From all these results, we can conclude that chicks need 7–10 μg. of biotin per 100 gm. of ration in addition to the amount found in our basal ration.

## SUMMARY

1. Biotin concentrates and free crystalline biotin have been found to prevent a typical dermatitis and to promote growth in chicks.

2. The biotin requirement of the chick is at least 7–10 μg. per 100 gm. of ration.

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# THE IRON REQUIREMENT OF CHILDREN OF THE EARLY SCHOOL AGE

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Although a number of studies have been made on the iron requirement of infants and preschool children, little is known concerning the requirement of the child of school age. It is the purpose of this study to make a contribution toward filling in this gap.

The method which has been used previously to determine the iron requirement has been that of the balance study. This method is a suitable one for finding the requirement of adults because the object is to find the lowest level at which the intake and outgo exactly balance. It is not necessarily applicable in the case of children, where the purpose has been to find an intake giving a large or even a maximum retention. It has been assumed that any excess beyond that needed to replace catabolic losses, and to furnish iron for growth as well as for a reasonable storage, would be excreted: that is, a child would not retain more iron than he needed. Of recent years experimental work has been done which indicates that this assumption was false.

Evidence that excess iron is not excreted was furnished by McCance and Widdowson ('38) who found that intravenous injections of iron in subjects on a constant iron intake resulted in practically no increase in fecal iron, and also by Hahn et al. ('39) who injected radio active iron in dogs and found that practically all the injected iron was retained, even though the dogs were so well stocked with iron that there was obviously no need for retention.

If excess iron is not excreted, large amounts will accumulate on high intakes. Such has been found to be the case by Hutchison ('37) who reported that when children were given large amounts of iron orally, all had large retentions as shown by balance experiments. The retention of one child in only 3.5 months was 3.5 gm., which is more than he could possibly have needed. Adults, too, have been shown to retain correspondingly large amounts on high iron intakes.

Since excess iron is not excreted, retentions may be much greater than the amount needed and there seems to be no way to tell how large retention should be. This being the case, it is necessary to find some criterion other than the size of the retention on a given intake for determining the amount of iron needed. Because a large proportion of the iron in the body is found in the hemoglobin, and because this is probably one of the first iron-containing tissues to be depleted when there is a deficiency, it would seem that a good hemoglobin level could be taken as an indication of an adequate iron supply. If so, the procedure would be to find the lowest level of iron intake that will produce and maintain a good hemoglobin level.

## EXPERIMENTAL

*The general plan.* Subjects were selected who had been living for several years in an institution where the diet was fairly uniform, in order that their hemoglobin levels could be considered a product of that diet. Their hemoglobin levels were determined and iron analyses were made on their diets. They were then matched in groups of three and given graded amounts of iron. After the iron supplementation had been continued for 7 months, hemoglobin levels were again determined and the data examined to find the lowest intake that gave good hemoglobin levels.

*The subjects.* This study was carried out at Mooseheart, an institution whose standards of nutrition and physical care are high. Twelve boys and nine girls, from the ages 8 to 11 years were selected as subjects. As a group, their nutritional condition rated higher than average. None of them had had

iron therapy or had suffered severe or chronic blood loss. All but one had been living on the diet of the institution for 2 years or more. It seemed, therefore, that their hemoglobins would be the product of that diet.

*The diet.* The diet was a good one containing all the foods customarily advised for children. Calculations made on the diets of the subjects showed that all were up to or above the daily allowances recommended by the Committee on Food and Nutrition of the National Research Council ('41) for every dietary essential. Probably, therefore, the diet furnished all the dietary factors which affect hemoglobin.

*The blood study.* Capillary blood was taken from the ear lobe. Hemoglobin determinations were made in duplicate by the method of Evelyn ('36) using the Evelyn Photoelectric Colorimeter. The red cells were counted from two pipettes of blood on a Bright-line Improved Neubauer Haemacytometer. The blood samples were taken during the time the dietary study was being made, on two different days, a week or two apart, always at the same time of day for the same child. The children were kept sitting quietly for at least 45 minutes before each test, and were kept as free as possible from excitement.

The individual hemoglobin values ranged from 13.8 to 11.6 gm. per 100 ml. of blood (table 1). The mean hemoglobin for all twenty-one children was 12.9 gm. The red cell counts ranged from 3,810,000 to 4,730,000 cells per cubic millimeter with a mean of 4,160,000 (table 2). There was no significant difference between the two sexes; the mean for the boys was 12.7 gm. of hemoglobin and 4,200,000 red blood cells per cubic millimeter and for the girls was 13 gm. and 4,120,000 cells. Since the analytical method was not accurate to more than 0.5 gm., all those hemoglobins falling between 12.5 and 14.5 gm. were within experimental error of the zone 13 to 14 gm., an area which is generally accepted as high normal.[1] Sixteen

[1] Evidence that the value 13–14 gm. of hemoglobin is a normal level for children in excellent health was obtained by an examination of the hemoglobin values of the state winners of the 4-H Club health contests. This information was made available by the Elizabeth McCormick Memorial Fund.

of the children fell in this zone; three fell in the range of 12 to 12.5 gm., which would still be considered normal; two fell in the range of 11 to 12 gm. which might be called low normal; and none even approached an anemic level which is usually considered as 10 gm. or below.

*The iron intake of the subjects.* The dietary study extended over a period of 2 weeks. A period of this length was used because it had been found by previous studies, made in this laboratory (McClelland, '31), that this is the shortest period

TABLE 1

*The relationship of the average daily iron intake to the original hemoglobin level.*

| SUBJECT AND SEX | AGE | HEIGHT | WEIGHT | HEMOGLOBIN | TOTAL IRON | IRON PER KILOGRAM |
|---|---|---|---|---|---|---|
| | *yr.–mo.* | *cm.* | *kg.* | *gm.* | *mg.* | *mg.* |
| T.R.—M | 9– 5 | 137.3 | 29.4 | 13.8 | 14.6 | 0.50 |
| C.W.—F | 10–10 | 143.4 | 33.4 | 13.6 | 11.4 | 0.35 |
| B.H.—M | 9– 0 | 131.8 | 32.2 | 13.5 | 14.8 | 0.46 |
| B.C.—M | 10– 0 | 137.0 | 32.6 | 13.4 | 12.5 | 0.38 |
| D.S.—F | 10– 4 | 140.3 | 33.4 | 13.3 | 11.6 | 0.35 |
| G.Ho.—F | 10– 9 | 141.5 | 37.7 | 13.3 | 11.7 | 0.31 |
| B.Co.—F | 10– 1 | 135.0 | 31.6 | 13.2 | 11.9 | 0.38 |
| R.C.—F | 9– 2 | 136.8 | 31.2 | 13.1 | 10.8 | 0.35 |
| H.S.—F | 10– 5 | 135.6 | 39.0 | 13.1 | 12.0 | 0.31 |
| J.J.—M | 8– 8 | 130.4 | 28.6 | 13.0 | 12.8 | 0.45 |
| B.W.—F | 9– 4 | 142.3 | 31.7 | 13.0 | 11.7 | 0.37 |
| A.H.—F | 11– 3 | 147.5 | 43.3 | 13.0 | 13.0 | 0.30 |
| G.H.—M | 8– 3 | 130.9 | 28.2 | 12.9 | 12.6 | 0.45 |
| G.B.—M | 9– 2 | 128.8 | 26.2 | 12.9 | 13.1 | 0.50 |
| R.J.—M | 9– 8 | 134.9 | 33.4 | 12.7 | 14.6 | 0.44 |
| A.Z.—M | 9– 4 | 137.2 | 32.2 | 12.5 | 11.3 | 0.35 |
| L.P.—M | 9–11 | 139.0 | 35.8 | 12.4 | 14.8 | 0.41 |
| C.R.—M | 9– 3 | 134.9 | 32.4 | 12.2 | 15.1 | 0.46 |
| E.D.—M | 9– 7 | 130.8 | 28.2 | 12.0[1] | 11.9 | 0.42 |
| A.P.—F | 9– 7 | 135.9 | 31.5 | 11.7 | 11.1 | 0.35 |
| R.H.—M | 10– 1 | 126.8 | 25.5 | 11.6 | 11.2 | 0.44 |
| Ave. M and F | 9– 9 | 136.1 | 32.3 | 12.9 | 12.6 | 0.40 |
| Ave. M | 9– 4 | 133.3 | 30.4 | 12.7 | 13.3 | 0.44 |
| Ave. F | 10– 2 | 139.8 | 34.7 | 13.0 | 11.7 | 0.34 |
| H.S.[2] | 10– 5 | 135.6 | 39.0 | 13.1 | 11.4 | 0.29 |

[1] Later rose to 13–14 area.

[2] For 12-week period.

that will furnish an accurate sample of a subject's customary intake. The food each child ate was weighed on Hanson spring scales to an accuracy of 1 gm. The subjects drank from thermos bottles filled with measured amounts of tap

TABLE 2

*The effect of iron administration for 7 months on the red cell counts and hemoglobin values.*

| | HEMOGLOBIN | | | RED CELLS | | |
|---|---|---|---|---|---|---|
| | Grams per 100 ml. | | | Millions per c.m.m. | | |
| | Spring | Fall | Change | Spring | Fall | Change |
| 0 mg. added iron | | | | | | |
| Boys T.R. | 13.8 | 14.3 | +0.5 | 4.73 | 4.34 | −0.39 |
| B.C. | 13.4 | 14.1 | +0.7 | 3.97 | 4.27 | +0.30 |
| J.J. | 13.0 | 13.8 | +0.8 | 4.41 | 3.97 | −0.44 |
| C.R. | 12.2 | 12.0 | −0.2 | 4.11 | 3.85 | −0.26 |
| Girls B.Co. | 13.2 | 13.0 | −0.2 | 3.84 | 3.86 | +0.02 |
| H.S. | 13.1 | 14.2 | +1.1 | 4.51 | 4.30 | −0.21 |
| A.H. | 13.0 | 13.0 | 0.0 | 4.10 | 3.75 | −0.35 |
| Average | 13.1 | 13.5 | +0.4 | 4.24 | 4.05 | −0.19 |
| 2 mg. added iron | | | | | | |
| Boys B.H. | 13.5 | 12.8 | −0.7 | 4.45 | 4.51 | +0.06 |
| G.H. | 12.9 | 12.5 | −0.4 | 3.98 | 3.86 | −0.12 |
| E.D. | 12.0 | 12.7 | +0.7 | 3.97 | 4.23 | +0.26 |
| L.P. | 12.4 | sick | | 4.40 | sick | |
| Girls G.Ho. | 13.3 | 13.7 | +0.4 | 4.18 | 4.18 | 0.00 |
| R.C. | 13.1 | 13.4 | +0.3 | 4.46 | 4.10 | −0.36 |
| B.W. | 13.0 | 13.2 | +0.2 | 3.91 | 3.79 | −0.12 |
| Average | 12.9 | 13.0 | +0.1 | 4.19 | 4.14 | −0.05 |
| 4 mg. added iron | | | | | | |
| Boys G.B. | 12.9 | 12.3 | −0.6 | 4.44 | 4.06 | −0.38 |
| R.J. | 12.7 | 13.3 | +0.6 | 3.81 | 4.22 | +0.41 |
| A.Z. | 12.5 | 12.9 | +0.4 | 4.15 | 4.14 | −0.01 |
| R.H. | 11.6 | 11.7 | +0.1 | 3.96 | 3.78 | −0.16 |
| Girls C.W. | 13.6 | 13.2 | −0.4 | 4.04 | 3.96 | −0.08 |
| D.S. | 13.3 | 13.1 | −0.2 | 4.00 | 3.95 | −0.05 |
| A.P. | 11.7 | 12.3 | +0.6 | 4.01 | 3.86 | −0.15 |
| Average | 12.6 | 12.7 | +0.1 | 4.06 | 4.00 | −0.06 |
| Average of all subjects | 12.9 | 13.1 | +0.2 | 4.16 | 4.05 | −0.10 |

water. Weekly composites were made of 1/10 aliquots of the food weighed on trip scales to an accuracy of 1/10 gm., and 1/10 aliquots of water were added.

The composites were digested with concentrated hydrochloric acid on a steam bath. Samples taken from the digests were wet-ashed. Iron was determined on an aliquot of the ash solution by a modification of Stugart's thiocyanate method ('31), with the use of an Evelyn Photoelectric Colorimeter. All containers were rinsed with dilute hydrochloric acid and triple-distilled water, and all possible precautions taken to avoid contamination.

The results of the iron analyses are shown in table 1. It will be noted that the total intakes ranged from 10.8 to 15.1 mg. with a mean of 12.6 mg. The mode, however, lay in the group below the mean, nine out of twenty-one children having intakes between 11.0 and 11.9 mg. and one being only slightly lower (10.8). In general the intakes of the boys were higher than those of the girls; the respective mean intakes were 13.3 and 11.7 mg. or 0.44 and 0.34 mg. per kilogram. The higher intake of the boys is interesting in view of the fact that they had neither a higher hemoglobin nor a higher cell count than the girls.

That the diet during the 2 weeks of the study was typical not only of the present diet but also of the one that had produced the hemoglobin level of the subjects, was borne out by three lines of evidence.

1. A chemical analysis of the diet of one of the girls for 10 additional weeks from April 6th to June 15th showed daily mean intakes of 10.5, 11.4, 10.9, 11.5, 11.2, 10.8, 11.2, 11.4, 12.5, 11.6 mg. with a mean for all 12 weeks of 11.4 mg. This was very near 11.7 mg., the mean for all the girls of the study.

2. Calculations were made from the dietitian's records of food supplied to each of the three cottages in which the subjects lived, for each month of the year, 1939–1940, including the months in which the 2-week samples were taken. The values for the 2 months in which the study was made were

14.5 and 15.5 mg. per day, values which were similar to those for the other months, and to the yearly average of 15 mg.

3. It happened, also, that records were available from a dietary study made in the same institution in 1932. They were compared with the calculated values found in the present study (1940) to determine whether the iron content of the diet had been the same for the last few years. Both of these computations were made from weights taken at the time the food was served on the plates. The mean for the 1932 group was 12.1 mg. and for the 1940 group, 12.6 mg.

Thus all three lines of evidence showed that the 2-week sample was surprisingly typical of the diet on which the children had been living.

*The iron supplementation.* The object in giving the iron supplements was to see whether the hemoglobin levels of any of the children would rise. A rise would indicate that the amount of iron originally in the diets was too small. The size of the supplement needed to bring about a rise would furnish information for estimating the minimum requirement.

The children having about the same hemoglobin values and red cell counts and approximately the same weight were arranged in groups of threes. One from each group was given 4 mg. of iron in addition to that in the diet, one 2 mg., and the third child none. These amounts were such that the total iron intakes per day would not be greater than could be obtained from foods. The supplement was given as the iron salt, ferric pyrophosphate. It was mixed with the milk in order to parallel the physical relationship of the iron in foods as nearly as possible. This salt was selected because it had been found by other workers to give good absorption when mixed with milk. This iron therapy was continued for 7 months because the amount of additional iron was so small that a long period was necessary to allow for a slow accumulation of new hemoglobin. At the end of this period, hemoglobin and red cells were again determined by the same methods and under the same conditions as at the beginning.

The results, as given in table 2, show that no significant change occurred in the blood picture, not even in the case of the subjects whose hemoglobin values were a little low. The hemoglobin would have had to rise as much as 1 gm. to be certain of a real change. In only one case was there as great a change as 1 gm. and that child was one of the controls who had no additional iron. The average for the whole group gave no indication of a tendency of the hemoglobin to rise. Only small and insignificant changes were found in the red cell counts. The supplementation, therefore, yielded no information as to the lowest iron intake needed for the formation of a good hemoglobin level but did show that the children were getting at least enough iron before the supplement was added.

*The lowest intakes found to be sufficient.* Since the real object of the whole study was to find the lowest level of iron intake that would support a good hemoglobin level, the data should be examined with this in mind. The subjects have been arranged in the order of the descending amounts of hemoglobin in table 1. The lowest amount of iron in the diet of a subject with a hemoglobin level of 12 gm. or higher was 10.8 mg. per day. This was but one case. That of six subjects averaged 11.4 mg. per day, which was exactly the average daily intake of the one subject who was studied for 10 additional weeks. It seemed justifiable, therefore, to consider this amount sufficient to cover the needs of children of this age.

Since, however, the children varied in age and size, the per kilogram values may have more significance. If all the subjects with a hemoglobin level of 12 gm. or more are considered, it will be observed that three of the children had good hemoglobin levels on approximately 0.30 mg. per kilogram. Since one of these was the child kept on the experiment for 12 weeks, and the only one showing a significant increase in hemoglobin level, it may be that 0.30 mg. per kilogram is sufficient. However, since these three children were all above the standard weight for children of their age, and would thus have a relatively high total intake on a low per kilogram intake, it might

not be wise to apply this figure to all children. The ten subjects with the lowest intakes were receiving 0.30 to 0.40 mg. per kilogram. Only two of these were boys. However, if two boys could build a good hemoglobin level on this amount, the rest could probably have done so. The mid-value of 0.35 mg. per kilogram would therefore appear to be an adequate allowance. If this value is applied to all the children of the study, it gives an average total iron of 11.3 mg. This is almost identical with the average total (11.4 mg.) of the six subjects with the lowest intakes and good hemoglobin values.

## DISCUSSION

Intakes of 11.4 mg. or 0.35 mg. per kilogram were apparently sufficient for the formation of a good hemoglobin level, but were they actually larger than necessary? This question cannot be answered with certainty until accurate studies have been made at lower intakes than these. Nevertheless it is possible to find some information on this point. The view that a lower intake would have sufficed is supported by the study of Davidson et al. ('33) who made a study among large groups of people from the poorer classes in Scotland. Calculations of the iron content of the diets were made from the foods entering the homes. Intakes of iron as low as 5 to 6 mg. "per man" (no doubt less per child) were found among families in which there was no anemia. In a study of 3000 individuals, Davidson and Fullerton ('38), also, found considerable anemia among very young children and women but little among men and children of the early school age. Since these people came from the poorer classes, the diets of the children undoubtedly contained less iron than that found in the diets of the subjects of the present study. The findings of Ross and Summerfeldt ('36) are to the contrary. They made a study on children 5 to 14 years of age living in an orphanage. The average hemoglobin for the group was low (10.2 gm.) although the diet, as calculated, contained 11 mg. of iron. Since the diets of their subjects were not as good as those of the present study and since supplementation with vitamins

$B_1$ and $B_2$ improved the hemoglobin of their subjects, it seemed likely that their diets may have been deficient in factors other than iron necessary for hemoglobin formation. Such experimental evidence as exists, therefore, points to the probability that the iron requirement as found by this study is higher than it needs to be.

It may be justifiable to try to get some idea of the minimum requirement by calculations such as those which have been published by Heath and Patek ('37). They estimated the amount of iron which would have to be retained each year in order to furnish enough for a normal increase in tissue and blood from birth to the end of the period of growth (table 3).

TABLE 3

*Estimates of the iron requirements of boys[1] from 2 to 11 years based on the retention figures of Heath and Patek.[2]*

| AGE | WEIGHT | RETENTION PER YEAR | INTAKE PER YEAR | INTAKE SMOOTHED | INTAKE PER DAY | INTAKE PER KILOGRAM |
|---|---|---|---|---|---|---|
| *yr.* | *kg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* |
| 2 | 13.4 | 80 | 800 | 850 | 2.3 | 0.17 |
| 3 | 15.3 | 92 | 920 | 920 | 2.5 | 0.16 |
| 4 | 17.3 | 98 | 980 | 980 | 2.7 | 0.15 |
| 5 | 19.6 | 79 | 790 | 1050 | 2.8 | 0.14 |
| 6 | 22.2 | 80 | 800 | 1120 | 3.1 | 0.14 |
| 7 | 24.6 | 70 | 700 | 1180 | 3.2 | 0.13 |
| 8 | 27.9 | 72 | 720 | 1250 | 3.4 | 0.12 |
| 9 | 30.9 | 152 | 1520 | 1320 | 3.6 | 0.12 |
| 10 | 34.5 | 130 | 1300 | 1390 | 3.8 | 0.11 |
| 11 | 37.6 | 137 | 1370 | 1450 | 4.0 | 0.11 |

[1] Retentions for girls are approximately the same.

[2] Heath, C. W., and A. J. Patek, Jr. 1937. The anemia of iron deficiency. Medicine, vol. 16, p. 279.

In order to find the size of the intake necessary to obtain a given retention, reference has been made to the percentage of retention found in balance experiments on children by the method of iron analysis used in this study.

Ascham ('35) and Oldham et al. ('37) found retentions of about 10%. Porter ('41) found retentions of about 20%. With the use of the polarographic method of analysis, Macy ('41) reported retentions of approximately 10%. Since it

is safest to use the percentage which would require the highest intake, a retention of 10% has been assumed for purposes of calculation. The required retentions as computed by Heath and Patek have then been multiplied by 10 to find the intake necessary to produce them and the values have been smoothed by inspection. By this method of calculation, a child of 10 would need a total of 3.8 mg. per day or 0.11 mg. per kilogram (see table 3). The intakes of all the subjects of the present study were much higher than this.

However, children of these ages need not only enough iron for maintenance and growth but also enough to allow the storage of iron in the liver and other storage depots. If the values given in table 3 are increased by 100% on the assumption that most of the stores must be accumulated in these years, the intake would be 7.6 mg. or 0.22 mg. per kilogram at 10 years. Thus even with allowances of 90% for loss in absorption and 100% for storage, the calculated iron requirement is still below the intakes found in the present study. It is true that figures such as these calculated ones, backed by no experimental work, cannot be used with much confidence but they do perhaps give some indication that the children of this experiment were getting even more iron than they needed for maintenance, growth and storage.

It may be of interest to compare the intakes of 0.35 mg. per kilogram found sufficient for the children of this study with those previously found for preschool children by means of the balance experiment. The requirements suggested have ranged from 0.60 mg. per kilogram (Rose et al., '30; Daniels and Wright, '34; Ascham, '35) to 0.48 mg. (Leichsenring and Flor, '32). However, since Porter ('41) has recently reported good retentions on intakes averaging 0.31 mg. per kilogram, it may be that the upper value of 0.60 mg., which has generally been accepted, is higher than it needs to be.

## SUMMARY

The purpose of this study was to find the iron requirement of children of the early school age. This was to be accom-

plished by finding the lowest iron intake which would support a good hemoglobin level.

Hemoglobin determinations were made on twenty-one children 8 to 11 years of age. Of these, nineteen had hemoglobin levels of 12–14 gm., the range considered normal.

The iron intakes were found by making a chemical analysis of the diet of each child over a 2-week period. An examination of the intakes indicated that good hemoglobin levels could be maintained on 11.4 mg. per day or 0.35 mg. per kilogram when the rest of the diet was adequate.

That a higher intake was not needed was shown by the fact that the hemoglobins of children given 2 or 4 mg. of iron daily over a 7-month period did not rise. It is possible, also, that lower intakes, had they been tried, might have been found to serve just as well.

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# THE ASCORBIC ACID REQUIREMENTS OF CHILDREN

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ONE FIGURE

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The practical problem of estimating the adequacy of the dietary with respect to ascorbic acid depends upon the knowledge of two factors: the ascorbic acid requirements and the amounts of ascorbic acid that various foods contribute after being prepared for consumption.

Since the development of chemical methods for ascorbic acid analysis it has been shown that in most normal subjects both the ascorbic acid value of the blood plasma and the amount excreted in the urine after a test dose reflect the previous dietary intake (Hawley, Stephens and Anderson, '36; Sendroy and Schultz, '36; Abbasy, Harris, Ray and Marrack, '35; Abt and Farmer, '36; Wright, Lilienfeld and MacLenathen, '37). Numerous reports have appeared in which either urine or blood analyses have been used to determine the state of ascorbic acid nutrition (Minot, Dodd, Keller and Frank, '40; Parran, '40). In a few cases studied, mostly those on adults, blood plasma determination or urinalysis has been supplemented by a determination of the intake in order to gain information on requirements (Belser, Hauck and Storvick, '39; Todhunter and Robbins, '40; Goldsmith, Ogaard and Gowe, '41; Smith, '36; Ralli, Friedman and Sherry, '39). However, the important problem of the ascorbic acid requirements for children has received less attention. Everson and Daniels ('36) in reporting a study on three pre-school boys,

considered 6.0 to 7.5 mg. per kilogram of body weight to be required daily for optimal retention. Widenbauer ('37) found that a daily intake of 22 mg. was a satisfactory level for a child 2½ years old. Hathaway and Meyer ('41) reported 31 mg. daily as sufficient to maintain "tissue saturation" in four cases from 4 to 5 years of age. These studies were based on the procedure of finding the individual daily ascorbic acid intake which would cause most of a subsequent test dose to be excreted in the urine. The individual intake was established either by direct food analysis or by the use of pure ascorbic acid.

Determinations of requirements by this method must, of necessity, be done on a small group of patients who are kept under the strict conditions of metabolism studies. Such careful physiological studies are essential, and undoubtedly when sufficient data of this type have been accumulated it will be possible to determine the requirements with considerable precision. However, in the application of such knowledge to the practical problems of human feeding, there will still exist the uncertainties involved in meeting these requirements with food. These uncertainties are many in the case of ascorbic acid, owing to the variable losses of this unstable substance, depending upon the type of food, conditions of growth, and the methods of marketing and preparation (Bessey, '38 b). The important practical problem in ascorbic acid nutrition is: How much of what kinds of food as usually prepared and consumed is necessary in order to be sure that the ascorbic acid requirements (whatever they may be) are adequately met? Because of the difficulties mentioned above, it seemed important to establish the ascorbic acid requirements for children under home conditions in terms of the food usually consumed, and by methods applicable in a nutrition clinic.

In this investigation, the blood plasma ascorbic acid levels of ninety-three healthy city children who were receiving variable amounts of ascorbic acid in their food, have been determined. Requirements were estimated by plotting these analyses against the dietary intakes as derived from dietary

histories considered unusually reliable, and locating the minimum intake which would give the average maximum post-absorptive blood plasma level.

## EXPERIMENTAL

Children attending the medical-nutrition clinic of the Forsyth Dental Infirmary were used for the study. This clinic provides periodic medical examinations and nutrition conferences for the children of the Infirmary as a means of improving dental conditions and promoting bodily health. Children are frequently under supervision for a period of several years. Since only those children who are financially eligible for clinic care are admitted to the Infirmary, the families represented in the study were in the lower income brackets of the community. Many were receiving assistance from public or private relief agencies.

By the system of including in the study the first few children who appeared on each clinic day, a representative cross-section was selected for blood determinations. The age range was from 5 to 13 years inclusive and approximately an equal number of boys and girls were used. Since all the children selected were given medical examinations on the day of the test, it was possible to exclude those who showed evidence of colds or other acute infection.

Specimens of blood were obtained by ear puncture with a Bard-Parker blade 4 to 24 hours after the last intake of a potent source of ascorbic acid (citrus fruits and tomatoes) and the analysis was made immediately. In many cases, subsequent blood plasma determinations were made after a lapse of a month or longer.[1]

The microphotoelectric indophenol method described by Mindlin and Butler ('38) with the modifications in manipula-

[1] Blood plasma analysis is preferred to urine determinations, especially for an out-patient clinic, since a satisfactory specimen may be obtained in a few minutes. One objection to its use for large groups of children has been the necessity of a venous puncture in order to obtain sufficient blood for an accurate analysis. This difficulty may be avoided by the use of the microphotoelectric technique, which requires only 0.25 cc. of blood. This amount may be easily obtained from the ear lobe without frightening the child.

tion and calculations suggested by Bessey ('38 a) for the macro method, was used for analysis. The acid filtrates and dye were measured in Lang-Levy pipettes (Levy, '36).

The values for the consumption of ascorbic acid were based on dietary histories secured by personal clinic interviews with the mother and child. The nutrition teaching that is regularly carried on in the clinic conferences was continued as usual, but for the purpose of the survey special attention was directed to the ascorbic acid consumption of the children from whom a blood sample had been taken. The mother and child were questioned minutely as to the food eaten during the 24-hour period previous to the clinic visit. Habits with respect to the usual consumption of the following foods were recorded: citrus fruits (oranges, grapefruit, tangerines and lemons) fresh or canned; citrus fruit juices, fresh or canned; tomatoes, fresh or canned; other fresh fruits and vegetables; and other canned or cooked fruits and vegetables. Detailed consideration was also given to the many factors which influence the child's consumption of these foods, such as family habits of purchasing, preparing and serving, food expenditures, meal planning, individual food preferences, and child management.

An estimate was made of the amount of ascorbic acid consumed daily by each child. The confidence with which the dietary data are regarded rests partially upon the fact that they were collected by a nutrition specialist in a clinic in which nutrition procedures were well established. Another significant feature is that many of the children had been under supervision for several years. In these cases, the records indicated clearly the response of the mother and child to clinic teaching and the validity of their statements. The trend of the child's dietary habits could therefore be appraised with considerable assurance.

Any record that contained an inconsistency or a suspicion of unreliability was discarded for the purpose of this report. From 180 cases, 93 with dietary records that were regarded as unimpeachable were selected.

Upon studying the dietary records, it was found that either citrus fruits or tomatoes constituted the principle year-around source of ascorbic acid for these children. The amount of ascorbic acid obtained from other sources appeared to be so small as not to influence the blood value, at least in the zone of concentrations found after liberal intakes — a point which will be discussed later. The estimate of the intake was, therefore, based on the consumption of citrus fruits and tomatoes only. Oranges, grapefruit and lemons were assumed to be equivalent. Tomatoes and tangerines were considered to contain about one-half as much vitamin C as oranges. The dietary estimates were made in terms of ounces of orange juice and the ascorbic acid calculated at 15 mg. per ounce.

## CONCLUSIONS AND DISCUSSION

Figure 1 shows the results of the blood plasma analysis for each level of ascorbic acid intake expressed as milligrams per day. One hundred and thirty-nine analyses on ninety-three cases are represented, since in several individual cases more than one determination was made.

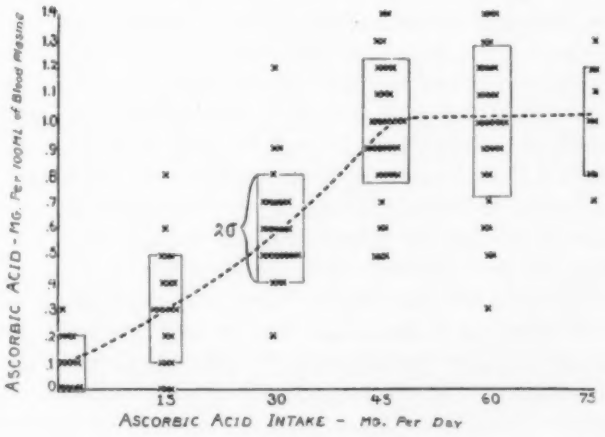


Figure 1

It will be noted that there is considerable scattering of points at any one level. This indicates a rather wide individual dispersion in the effect of a given quantity of orange juice on the blood plasma ascorbic acid level, and therefore suggests the caution which is necessary in evaluating the state of ascorbic acid nutrition of an individual by this method; but, in spite of this variation, there is a clear tendency for the points to concentrate around certain values.

It is an interesting fact that a curve drawn through the average values duplicates the curves that have been obtained by one of us (O.A.B.) when repeated analyses have been made on normal individuals on gradually increasing doses of pure ascorbic acid. This agreement in the data collected by two different methods gives support to the validity of the data presented. In figure 1, it will be observed that up to the level of about 3 ounces of orange juice, the ascorbic acid value of the blood plasma shows a gradual increase as the intake of ascorbic acid increases. Beyond this point the increase in value is slight, if any. Further support is given to the reliability of the data by the fact that this maximum concentration is the value found by kidney threshold investigations (Ralli, Friedman and Sherry, '39).

The data presented indicate that 3 ounces of orange juice per day, or its equivalent, plus the small amount of ascorbic acid obtained in other foods, which probably totals not more than 10 mg. per day in this group of children, is sufficient to produce an optimal post-absorptive plasma ascorbic acid concentration for the average child aged 5 to 13 years. Assuming orange juice to contain 15 mg. of ascorbic acid per ounce, the requirements may be placed at 40 to 50 mg. daily. It is true that on this level of intake some cases do not reach the average optimal blood plasma value. However, the number of such cases falling below the standard seems also to be about the same on both the 4-ounce and the 5-ounce intake. That this low level may be characteristic of the individual is suggested by the fact that reanalyses several weeks apart checked each other almost without exception within the limits of the method.

This finding also corresponds with the experience of one of us (O.A.B.) with certain other individuals who fail to reach the expected fasting blood plasma level even when the intake of pure ascorbic acid is very high.

When not only the citrus fruits and tomatoes but also all other fruits and vegetables were included as sources of ascorbic acid, no correlation was apparent between the dietary evaluation and the blood plasma values. Since there is no evidence to indicate that ascorbic acid from sources other than citrus fruits and tomatoes is poorly utilized, we must interpret this lack of correlation as meaning that our estimates of the amounts of ascorbic acid contributed by fruits and vegetables other than the citrus fruits and tomatoes were incorrect. We believe that this difficulty often arises from the use of food-value tables for the ascorbic acid content of foods which are invariably subject to large and unpredictable losses during marketing and preparation.

That the amount of ascorbic acid obtained from sources of low potency has little influence on blood plasma values is indicated also by the fact that when children on the same levels of citrus fruit intake were divided into two groups, namely those receiving the largest amount of other types of fruit and vegetables and those receiving the smallest amounts, there was no difference in the average blood plasma values.

Eight children of the group studied consumed practically no citrus fruits or tomatoes for several months during the study. The blood plasma values of these children were either zero or near zero on every analysis. Four cases failed to show ascorbic acid in the blood plasma at any time, yet clinical evidences of scurvy were absent. This fact seems to indicate that for such a group the average daily consumption of ascorbic acid from sources other than citrus fruits and tomatoes is sufficient to prevent scurvy, but insufficient to give satisfactory blood plasma levels. The absence of scurvy in these cases confirms several recent reports (Butler and Cushman, '40; Crandon, Lund and Dill, '40; Milam and Wilkins, '41) that zero or low plasma levels are not necessarily indicative of this

disease. Under such conditions, the tissues although probably handicapped, may receive enough ascorbic acid to prevent the development of pathology.

We do not wish to leave the impression that food sources other than citrus fruits and tomatoes are unimportant or unable to supply the ascorbic acid required to produce satisfactory plasma values. Obviously a sufficient intake of any ascorbic-acid-containing food should be adequate; and undoubtedly during certain seasons or in certain regions the intake of such products as strawberries and raw green leafy foods may be great enough to be sufficient. However, the average city child does not regularly consume enough of these foods to reach a desirable intake of ascorbic acid.

Our findings indicate that an estimation of the intake of citrus fruit and tomato serves as a very simple and satisfactory practical means of evaluating a diet for vitamin C when one is concerned with optimal nutrition, and just as the regular consumption of milk is considered a practical necessity to assure adequate calcium, so the regular use of citrus fruits or tomatoes is the most practical and reliable means of assuring the optimal ascorbic acid intake.

The point is often raised as to whether the placement of the requirement at the amount which produces the plasma concentration usually found after liberal intake may not be too high a standard. It seems likely that this level is above the optimal physiological requirement and that it involves some waste; but until methods are available for fixing more exactly the value of the physiological optimum, it would seem to be a good nutritional practice to recommend a daily intake of ascorbic acid that is within the range of the physiological maximum.

## SUMMARY

Blood plasma ascorbic acid analysis on ninety-three healthy city children whose ascorbic acid intake is evaluated from thorough dietary histories is presented.

Eighty per cent of the children who received 3 ounces or more of orange juice daily or an equivalent amount of ascorbic

acid in citrus fruits and tomatoes maintained a blood plasma level of ascorbic acid indicating a liberal intake. Those who received less than 3 ounces showed blood plasma values which were correspondingly lower. There were a few individuals whose blood plasma values remained definitely low in spite of a regular intake considered adequate for the average child.

The amount of ascorbic acid consumed daily from sources other than citrus fruits and tomatoes, that is, other fruits and vegetables, was insufficient in this group to influence the blood plasma values. This finding emphasizes the necessity of a regular consumption of citrus fruits or tomatoes if an optimal post-absorptive plasma value is to be maintained. Furthermore, it suggests that in taking nutritional histories the evaluation of the citrus fruit and tomato intake is a simple, practical, and accurate means of determining ascorbic acid consumption, at least as far as the optimum range is concerned.

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